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Overweight and obesity are the most widespread nutritional diseases in the U.S., which greatly increase chronic disease risks and mortality. Therefore, it is urgent to develop a relatively efficacious and safe strategy for the weight management. Consumption of conjugated linoleic acid (CLA) supplements or one of its isomers, trans-10, cis-12 (10,12) CLA, has consistently demonstrated reductions in body weight or body fat in human and animal studies.

Our lab has demonstrated that 10,12 CLA triggered calcium release from endoplasmic reticulum in human primary adipocytes, which activated downstream inflammatory signaling, resulting in impaired uptake of glucose and fatty acid, and delipidation. However, the upstream signals responsible for these actions are unknown. Therefore, my Aim 1 investigated the upstream mechanism by which 10,12 CLA increases intracellular calcium and inflammatory signaling in human primary adipocytes. The results indicated that phospholipase C plays an important role in 10,12 CLA-mediated activation of intracellular calcium accumulation, inflammatory signaling, delipidation, and insulin resistance in human primary adipocytes.

It has been demonstrated that 10,12 CLA increased mRNA levels and protein levels of cyclooxygenase-2 (COX-2) and pro-inflammatory prostaglandins, which have been linked to increased energy expenditure associated with white adipose tissue (WAT) browning and uncoupling of ATP synthesis. It also has been shown that relatively high doses of 10,12 CLA lead to more significant reductions in body fat, but cause a greater level of inflammation, insulin resistance, and steatosis in animals. Therefore, Aims 2 and

Aim 3 determined the extent to which a relative low dose of 10,12 CLA or a CLA isomer mixture increases markers of browning in mice and its dependence in inflammatory signaling. In Aim 2, a low threshold dose of 10,12 CLA was found that prevented body fat accumulation with minimum metabolic side-effects in non-obese mice. It was also found that 10,12 CLA-induced browning in WAT was accompanied by increases in mRNA levels of COX-2 and other markers of inflammation. In Aim 3, a relatively low dose of 10,12 CLA reduced body fat and increased browning of WAT in overweight mice, which were independent of inflammatory signaling.

Collectively, these findings provide critical insights for the development of reliable dietary strategies for people who take CLA as method to lose weight. However, we still do not know (i) if 10,12 CLA supplementation would effectively reduce body fat in overweight mice when they are continuously fed an American-type, high-fat diet; (ii) potential risks of impaired regulation of body temperature, inflammation, and steatosis due to 10,12 CLA consumption in high fat-fed mice; and (iii) potential mechanisms by which 10,12 CLA reduces body fat in high fat-fed mice.

ANTI-OBESITY MECHANISMS OF CONJUGATED LINOLEIC ACID (CLA): ROLE OF INFLAMMATORY SIGNALING AND BROWNING IN WHITE ADIPOSE TISSUE

by

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CHAPTER I

INTRODUCTION

Overview

Obesity is the most widespread nutritional disease in the U.S. (1). Excess body fat increases risks of several chronic diseases and mortality (2), which causes a great economic burden. Many Americans are consuming dietary supplements for fat loss including conjugated linoleic acid (CLA), which contains an equal mixture of cis-9, tran-11 (9,11) and trans-10, cis-12 (10,12) CLA isomers. Consumption of CLA supplements or 10,12 CLA alone has consistently demonstrated reductions in body weight or body fat in at least 30 animal and 15 human studies (reviewed in 3). Proposed anti-obesity mechanisms of CLA, specifically 10,12 CLA, include regulation of 1) energy metabolism, 2) adipogenesis, 3) lipid metabolism, 4) inflammation, and 5) adipocyte apoptosis (reviewed in 4).

Our lab has previously demonstrated that 10,12 CLA activated diacyglycerol kinase (5), which triggered calcium release from endoplasmic reticulum (6) in human primary adipocytes. This accumulation of intracellular calcium resulted in up-regulation of downstream extracellular signal-regulated kinase (ERK)1/2 (7, 8), cJun-NH2-terminal kinase (9), nuclear factor kappa B (NFκB) (10), and prostaglandin synthesis (6, 11). These inflammatory signals were associated with reductions in the abundance and activity of peroxisome proliferator-activated receptor γ and CAAT/enhance binding protein α, and suppressed their target genes (7-10, 12). These alterations were associated with impaired uptake of glucose and fatty acids (11) and contributed to the

reduction of lipid content (6, 12) in adipocytes. However, the upstream mechanism(s) by which 10,12 CLA increases intracellular calcium and inflammatory signaling is unknown, and will be the focus of Aim 1 of my dissertation research.

It has been shown that the higher the dose of CLA used, the more significant loss of body fat in animals (13). However, relatively high doses of 10,12 CLA or the mixture of CLA isomers caused a greater level of inflammation, insulin resistance, and steatosis (14-16). Therefore, my Aim 2 will determine if a relative low dose of 10,12 CLA or the CLA mixture reduces body fat with minimum side-effects.

Our lab demonstrated that 10,12 CLA increased mRNA levels and protein levels of cyclooxygenase-2 and two pro-inflammatory prostaglandins (11), which have been linked to increased energy expenditure associated with white adipose tissue (WAT) browning and uncoupling of ATP synthesis (17). Therefore, Aims 2 and Aim 3 will also determine the extent to which a relative low dose of 10,12 CLA or the CLA mixture increases markers of browning in mice and its dependence in inflammatory signaling. The Aim 3 will also determine if daily exercise prevents 10,12 CLA-mediated steatosis.

Central Hypothesis and Specific Objectives

The *long-term goal* of this project is to develop efficacious and safe strategies for the control of human obesity. The *central hypothesis* of my dissertation research is that 10,12 CLA decreases body fat loss by promoting 1) upstream inflammatory signals that generate lipid-borne metabolites that antagonize transcription factors regulating lipid metabolism, thereby inhibiting *de novo* adipogenesis and lipogenesis in adipocytes, and 2) browning in WAT, thereby increasing energy expenditure in vivo. The rationale that underlies the proposed studies is that by establishing how 10,12 CLA influences

metabolism and identifying potential side effects, a reliable strategy for people who take CLA as an option for weight loss can be obtained by magnifying its benefits and eliminating its potential adverse side effects.

In order to achieve this long-term goal, I will examine the central hypothesis and attain the overall objective of this project by pursing the following specific aims: Aim 1) Identify upstream mechanisms by which 10,12 CLA regulates inflammatory signaling and lipid metabolism in adipocytes; Aim 2) Identify a threshold dose and isomer of CLA that prevents body fat accumulation with minimum metabolic side-effects in non-obese mice, and its impact on markers of mitochondrial biogenesis, thermogenesis, and inflammatory signaling; and Aim 3) Determine the extent to which 10,12 CLA decreases body fat in overweight mice, its dependence on inflammatory signaling, and the synergistic effects of exercise.

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CHAPTER II

REVIEW OF THE LITERATURE

Obesity

Overweight (i.e., body mass index (BMI) between 25 and 30) and obesity (i.e., BMI greater than 30) are global nutritional diseases, affecting over one-third of the adult population (1). In 2008, 1.4 billion and 500 million adults worldwide were overweight and obese, respectively (1). Strikingly in the U.S., 68% of adults above age 20 were overweight, and 34% were obese in 2008 (2). Excess body fat increases chronic disease risks and mortality (3), For example, a BMI greater than 25 is accompanied by a 2 to 50-fold increase in the relative risks of cardiovascular diseases, diabetes, gallbladder diseases, renal diseases, and cancer (4). These diseases result in an estimated economic burden of $270 billion annually in the U.S. (4). Therefore, developing a long-term and safe strategy for the weight control is urgent.

Conjugated Linoleic Acid (CLA)

Many Americans are consuming dietary supplements for weight management, including CLA, which was given Generally Recognized as Safe status by the FDA in 2008. CLA generally refers to long chain, unsaturated fatty acids with 18 carbon atoms and at least one conjugated double bond. They are naturally produced by bacteria fermentation of linoleic acid in ruminant animals during digestion. Therefore, dairy products and ruminant animal meats are good natural sources for CLA. On average, people in the U.S. consume approximately 151-212 mg/d of CLA from their diets, which

contain approximately 90% cis-9, trans-11 (9,11) CLA isomer and 10% trans-10, cis-12 (10,12) CLA isomer (reviewed in 5, 6). Studies have demonstrated that 10,12 CLA is the isomer responsible for reducing body fat (reviewed in 5, 7-9). Commercial CLA supplements usually contain 1,000 mg CLA per pill, with a 50:50 mixture of the 9,11 and 10,12 CLA isomer (i.e., CLA mixture).

Anti-obesity Mechanisms of 10,12 CLA

In 1997, Park et al. were the first to report a significant reduction in body fat in mice fed a 0.5% CLA supplemented diet (10). Subsequently, the mixture of CLA isomers, or 10,12 CLA alone, consistently reduced body fat in rodents (Table 2.1, 11-20) and humans (Table 2.2, 21-30). Proposed anti-obesity mechanisms of 10,12 CLA include regulation of 1) adipogenesis, 2) lipid metabolism, 3) energy metabolism, 4) adipocyte apoptosis, and 5) inflammation (reviewed in 7).

The inhibition of adipogenesis and lipogenesis by 10,12 CLA is partially mediated by a reduction of mRNA, protein, or activity levels of peroxisome proliferator activated receptor (PPAR) γ (31-35) and CAAT/enhancer binding protein (C/EBP) α (32,33,35), two key transcription factors regulating adipocyte differentiation and lipid synthesis. The 10,12 CLA-mediated suppression of these two transcription factors resulted in decreased expressions of their target genes including acetyl CoA carboxylase (35), stearoyl-CoA desaturase-1 (33), fatty acid synthesis (33), and adipocyte fatty acid binding protein (32, 33, 35), all of which play important roles in lipid synthesis and accumulation. Notably, 10,12 CLA, but not 9,11 CLA, attenuated protein levels of perilipin and hormone-sensitive lipase in human primary adipocytes after 3 days of

treatment (36). It also has been shown that 10,12 CLA induced lipolysis and delipidation in vitro (36-37) and vivo (38-39) studies.

10,12 CLA-mediated increase in energy expenditure has been associated with increased oxygen consumption (40), induction of fatty acid oxidation (12, 20, 41) and uncoupling of mitochondrial respiration in WAT (13, 14, 20, 42) and muscle (43), increased lean body mass (25-26, 28), and energy loss in the excreta (44). However, controversy remains as to the extent to which 10,12 CLA affects energy intake. Several studies reported that 10,12 CLA suppressed energy consumption via impacting hypothalamic signaling or appetite in rodents (45-46) and human subjects (47). In contrast the majority of studies reported no impact of CLA supplementation on food intake (11-12, 48).

Some studies indicated that CLA decreased fat mass via increasing adipocyte apoptosis. 10,12 CLA mediated induction of adipocyte apoptosis could be due to mitochondria stress, tumor necrosis factor (TNF) α induction, or an integrated stress response pathway (reviewed in 7). Treatment with the CLA mixture (100 μM, 50:50 of 9,11 and 10,12 isomers) for 24 h induced apoptosis in 3T3-L1 adipocytes (49). In vivo, supplementation of the CLA mixture (1%, 50:50 of 9,11 and 10,12 isomers) elevated mRNA levels of several apoptotic related genes including those in the TNF family and cell death pathways in WAT depots of male C57BL/6J mice after 1 d of feeding (50). The same dose of CLA supplementation promoted apoptosis in WAT in female C57BL/6J mice (13) and mixed strains of mice (51) within 1 wk.

Collectively, these data indicate that 10,12 CLA reduces adiposity, in part, by 1) suppressing adipogenesis and lipogenesis, 2) increasing fatty acid oxidation and energy expenditure, and 3) causing adipocyte stress and apoptosis. Notably, it has been shown

10,12 CLA rapidly increased intracellular calcium levels via release from the endoplasmic reticulum (ER) , and reactive oxygen species production and MAPK cascades. Collectively, these activated signaling pathways lead to increased inflammatory responses (52), and interfered with adipogenesis (35, 53), lipid mobilization (35, 53-54), and insulin sensitivity (35, 53, 55). Therefore, inflammatory signaling appears to play an important role in 10,12 CLA-mediated reductions in WAT or adipocyte lipid content.

10,12 CLA and Inflammation

10,12 CLA triggers inflammatory signaling in adipocytes

Our lab has demonstrated that 10,12 CLA-mediated inflammatory signaling is due, in part, to ER stress and further driven by activation of mitogen-activated protein kinase/extracellular signal regulated kinase (MEK/ERK) and nuclear factor kappa-light-chain enhancer of activated B (NFκB) pathways (56-58). For example, 10,12 CLA caused an early (i.e., within 2 min) accumulation of intracellular calcium from the ER (58), followed by an induction of mRNA levels of activating transcription factor (ATF)3 within 4 h (59), as well as induction of calmodulin kinase II and X-box binding protein within 12 h (58-59), all of which are ER stress-related markers. Blocking calcium release from ER attenuated 10,12 CLA-mediated phosphorylation of ERK and c-Jun N-terminal kinase (JNK), expression of total ATF3, and binding activity of NFκB (58), suggesting that 10,12 CLA-activated ERK, activator protein (AP)-1, and NFκB signaling are dependent on intracellular calcium accumulation. These 10,12 CLA-induced inflammatory signaling further impaired PPARγ activity and abundance (35, 53, 60), which leads to reduced glucose and fatty acid uptake and *de novo* lipogenesis (35, 53-

54, 60). Our lab has also demonstrated that 10,12 CLA-mediated lipolysis and insulin resistance in adipocytes was NFκB and MAPK signaling-dependent (53, 55-56). Taken together, these data indicate that 10,12 CLA-mediated inflammation may be responsible, in part, for delipidating adipocytes. However, the upstream signal initiating these pro-inflammatory, lipid-lowering properties of 10,12 CLA are unknown. Recently, our group showed that 10,12 CLA increased the mRNA levels of several G-protein coupled receptors (61); and inhibition of 10,12 CLA-mediated diacyglycerol kinase (DGK) activity attenuated intracellular calcium accumulation and downstream inflammatory signaling (52). These findings advance that the upstream enzyme phospholipase c (PLC) may play an important role in 10,12 CLA-triggered inflammation in adipocytes, which will be the focus of Aim 1 (Figure 2.1).

10,12 CLA inflames WAT

10,12 CLA increases gene expression and protein secretion of inflammatory markers (56-58), and further inflames surrounding WAT via paracrine and autocrine signaling (53, 56) and activating immune responses (62-63). For example, mature primary human adipocytes, but not preadipocytes, responded to 10,12 CLA by increasing mRNA level of interleukin (IL)-6, IL-8, IL-1β, and cyclooxygenase (COX)-2, and secretion of IL-6, IL-8, prostaglandin (PG)E_2 and PGF_2 (56). Consistent with these in vitro data, 10,12 CLA supplementation of C57BL/6J mice increased the expression levels of IL-6, TNFα, and IL-1β in WAT, and macrophage infiltration and numbers of monocytes in the surrounding blood vessels (62-63).

Relationship between10,12 CLA-mediated inflammation and WAT browning

Recently, inflammation has been linked to WAT browning (64), i.e., increased mitochondria abundance, fatty acid oxidation, respiratory uncoupling, and heat production in WAT (65-66). COX-2, the rate-determining enzyme in prostaglandin synthesis (64, 67), and two of its products, PGE_2 and PGI_2 (68), were positively linked to mitochondrial biogenesis in WAT. Coincidently, treatment of human adipocytes with 10,12 CLA up-regulated mRNA levels of COX-2 and prostaglandin production (e.g. PGE_2 and PGF_2) (56-58), and markers of browning (e.g. uncoupling protein (UCP)-1) (14, 20, 42-43, 63, 69) and fatty acid oxidation (e.g. carnitine palmitoyltransferase (CPT)-1b) (20, 70-71) in WAT. However, we do not know the extent to which inflammation is the mediator or the consequence of 10,12 CLA browning in WAT, and the dose of CLA supplementation used in animals to reduce body fat with minimum side-effects. Therefore, answering these questions is the focus of Aims 2 and 3 (Figure 2.2).

Safety Concerns of CLA Supplementation

Delany et al. fed male AKR/J mice with the CLA mixture (50:50 of 9,11 and 10,12 isomers) at 0.25%, 0.5%, 0.75%, and 1.0% (w/w) and found a dose-dependent loss of body fat when given 0.4% to 1.0% of the CLA doses (48). These data indicated that the higher doses of CLA apply, the more significant fat loss could occur. In order to achieve this desired CLA-mediated reduction in body fat, many studies have used 1.0% dose of the CLA mixture or 0.5% dose of 10,12 CLA alone, or higher (11-15, 17, 72), which are 10-30 times (per kg body weight) higher than the dose used clinical trials. Metabolic side-effects such as impaired insulin function (13, 19, 72), steatosis (13, 19, 72), and decreased blood leptin levels (12) have been reported at this dose or higher (Table 3,

12, 13, 15, 18-19, 72-73). Recent rodent studies have been using as low as 0.3% of the CLA mixture (73-74) with minimal adverse effects. Unfortunately, no significant changes in body fat were found at this low dose. Therefore, the existence of a threshold dose of CLA that safely and effectively reduces body fat with minimum adverse side effects will be a great interest, and thus will be the focus of Aim 2 (Figure 2). We also would like to examine the synergistic effects of CLA supplementation and exercise in reducing body fat and preventing steatosis, which will be the focus of Aim 3 (Figure 2).

In randomized clinical trials, adverse symptoms such as constipation, diarrhea, myocardial infarction, musculoskeletal ailments, and stomach and gastrointestinal stress were reported among healthy overweight and obese subjects who were taking 3.4 g to 6 g of the CLA mixture daily for 6 to 12 month (reviewed in 75). In these studies, CLA did not affect waist circumference, but significantly decreased body weight by 0.7 kg, body fat by 1.33 kg, and BMI by 0.3 compared to the placebo groups. Consuming 3.4 g of the CLA mixture per day for 2 years significantly reduced serum leptin levels in human participants (76). Inflammatory markers C-reactive protein and aspartate transaminase were elevated in blood after consumption of 2.4 g to 6.4 g of the CLA mixture from 4 wk to 2 years (reviewed in 77). An individual case of a 46 year old female developing severe hepatitis accompanied with jaundice after 14 d self-medication of CLA (dose not reported) indicated potential safety concerns of applying CLA supplements (78). Therefore, understanding the mechanism by which CLA reduces body fat and identifying an effective dose of CLA that does not increase adverse side effects will provide important insights for determining if CLA can reduce body safely. Until such information becomes available, a safe and reliable use of this dietary approach to reducing or preventing increased adiposity cannot occur.

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Table 2.1 CLA Decreased Body Fat in Rodents

Model	Daily CLA dose (w/w) and two isomer ratio	Equivalent CLA dose as mg/kg body weight	Length of the study	Major effects in body composition	Reference
Male AKR/J mice, 4 wk old	1%, 1:1	1200	5 wks	↓ body fat, ↑ energy expenditure, no change in body weight and energy intake	11
Male OLEFT rats, 4 wk old	1%, 1:1	1200	4 wks	↓ WAT, ↓ body weight, no change in food intake	12
Female C57BL/6J mice, 7 wk old	1%, 1:1	1200	4 days to 32 wks	↓ WAT and ↓ BAT	13
Female C57Bl/6 mice, 5 wk old	1%, 1:1	1200	3 wks	↓ WAT	14
Male C57BL/6J and ICR mice	1%, 1:1	1200	21 d	↓ WAT	15
Male Balb/c mice, 6 wk old	0.5%, 1:1	600	14 wks	↓ body weight gain, ↓ body fat gain, ↓ visceral fat mass gain	16
Male inbred AKR/J mice , 6 wk old	1.2%, 1:1	1440	6 wks	↓ body weight, ↓ WAT weight (retroperitoneal being most sensitive), ↓ energy intake, ↑ metabolic rate	17
Male C57BL/6J mice, 6 wk old	1.5%, 1:1	1800	4 wks	↓ body weight gain, ↓ weights of epididymal and inguinal fat	18
Female C57BL/6 mice, 11 month	0.5%, 1:1	600	6 month	↓ body weight, ↓ WAT mass	19
SCD-1-null mice, 7 wk old	0.2% 10,12 CLA	240	4 wks	↓ body fat (epididymal and retroperitoneal fat)	20

Table 2.2 CLA Decreased Body Fat in Clinical Trials

Human Model (BMI)	Daily dose of the CLA mxiture (gram/d, 50:50)	Equivalent CLA dose as mg/kg body weight	Length of the study	Major effects in body composition	Reference
27.5-39	3.4	37.8	12 wk	↓ mean body weight by 1.1 kg, ↓ mean BMI by 0.4 kg/m^2	21
25-35	4 doses: 1.7, 3.4, 5.1, and 6.8	18.9, 37.8, 56.7, and 75.6	12 wk	↓ body fat mass in 1.7, 3.4, and 6.8 g/d groups, compared to the each placebo	22
25.5±3.9	4.2	54.5	12 wk	↓ body fat ($p<0.5$ compared to the baseline)	23
25-30	~3.6	45	48 wk	↓ body fat ($p<0.5$ compared to the baseline and the placebo group	24
~25	5	66.7	7 wk	↓ body fat and ↑ lean body mass ($p<0.5$ compared to the baseline and the placebo group)	25
28-32	3.4	40.5	48 wk	↓ body fat mass, and ↑ lean body mass ($p<0.5$ compared to the baseline and the placebo group)	26
25-30	3.2	40	48 wk	↓ body fat mass ($p<0.5$ compared to the baseline); helped maintain the weight from holiday eating	27
30-35	3.2 or 6.4	34	12 wk	↑ lean body mass in 6.4 g/d group ($p<0.5$ compared to the baseline).	28
postmen opausal women with T2DM; BMI > 30	6.4	65.3	36 wk	↓ BMI and ↓ body fat ($p<0.5$ compared to the baseline)	29
postmen opausal women; BMI < 35	5.5	78.6	16 wk	↓ total and lower body fat ($p<0.5$ compared to the placebo)	30

Table 2.3 Adverse Effects of CLA Supplementation in Rodents

Model	Daily CLA dose (w/w) and two isomer ratio	Equivalent CLA dose as mg/kg body weight	Length of the study	Major adverse effects from CLA	Reference
Male OLEFT rats, 4 wk old	1%, 1:1	1200	4 wks	↓ leptin in blood	12
Female C57BL/6J mice, 7 wk old	1%, 1:1	1200	4 days to 32 wks	↓ leptin in blood, induced insulin resistance, steatosis, and apoptosis adipocytes	13
Male C57BL/6J and ICR mice	1%, 1:1	1200	21 d	induced steatosis	15
Male C57BL/6J mice, 6 wk old	1.5%, 1:1	1800	4 wks	↑ liver weight	18
Female C57BL/6 mice, 11 month	0.5%, 1:1	600	6 month	induced fatty liver and insulin resistance	19
Female C57Bl/6J mice, age NA	0.4% pure 10,12 CLA or 9,11 CLA	480	4 wks	10,12 CLA isomer, not 9,11 CLA induced steatosis, hyperinsulinemia and lipoatrophy	72
Male C57BL/6J mice, 5 wk old	Dose 1: 0.3%, 1:1; Dose 2: 0.6%, 1:1	Dose 1: 360; Dose 2: 720	Phase 1: 30 d on dose 1; Phase 2: 35 d on dose 2	induced insulin resistance at 2nd phase	73

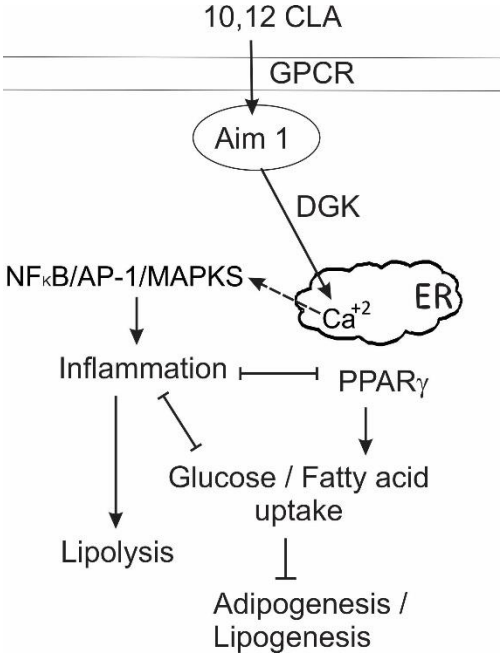

Figure 2.1 Research Aim 1. 10,12 CLA activates upstream signals which mediate downstream inflammatory signaling, lipolysis, impaired glucose and fatty acid uptake, and inhibition of adipogenesis and lipogenesis in human primary adipocytes (proposed mechanism in Aim 1)

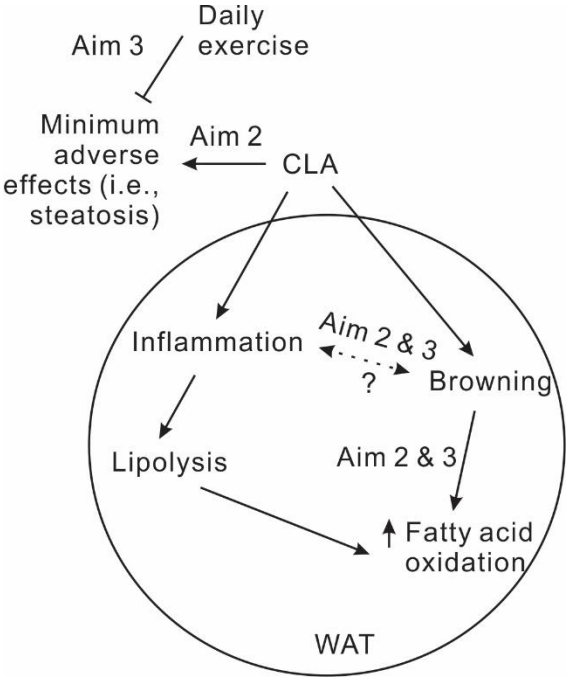

Figure 2.2 Research Aim 2 and Aim 3. Aims 2 and Aim 3 determined the extent to which a relative low dose of 10,12 CLA or a CLA isomer mixture increases markers of browning in white adipose tissue (WAT) in mice and its dependence in inflammatory signaling. Adverse metabolic effects of the CLA supplement (Aim 2) and the synergistic effects of exercise (Aim 3) were also investigated.

CHAPTER III

THE PHOSPHOLIPASE C INHIBITOR U73122 ATTENUATES TRANS-10, CIS-12 CONJUGATED LINOLEIC ACID-MEDIATED INFLAMMATORY SIGNALING AND INSULIN RESISTANCE IN HUMAN ADIPOCYTES

Formatted for Shen W, Chuang CC, McIntosh M. The phospholipase C cinhibitor U73122 attenuates trans-10, cis-12 conjugated linoleic acid-mediated inflammatory signaing and insulin resistance in human adipocytes (2013). *Journal of Nutrition, 143(5),* 584-90.

Abstract

We have demonstrated that trans-10, cis-12 conjugated linoleic acid (18:2t10,c12)-mediated delipidation of human adipocytes was dependent on increased intracellular calcium and activation of inflammatory signaling in human primary adipocytes. These data are consistent with the actions of diacylglycerol and inositol triphosphate derived from phospholipase c (PLC)-dependent cell signaling. To test the hypothesis that PLC was an upstream activator of these cellular responses to 18:2t10,c12, primary cultures of human adipocytes were pretreated with 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione (U73122), a universal PLC inhibitor, followed by 18:2t10,c12 treatment. U73122 attenuated 18:2t10,c12-mediated insulin resistance within 48 h and suppression of the mRNA levels of peroxisome proliferator-activated receptor (PPAR)γ, insulin-stimulated glucose transporter-4, acetyl-CoA carboxylase-1, and stearoyl-CoA desaturase-1, and the protein levels of PPARγ within 18–24 h. U73122 inhibited 18:2t10,c12-mediated induction of the inflammatory-related genes calcium/calmodulin-dependent protein kinase-β, cyclooxygenase-2, monocyte chemoattractant protein-1, interleukin (IL)-6, and

IL-8, secretion of IL-6 and IL-8, and the activation of extracellular signal-related kinase, c-Jun N-terminal kinase, and c-Jun within 18–24 h. Moreover, 18:2t10,c12 increased the mRNA levels of heat shock proteins within 6–24 h and intracellular calcium concentrations within 3 min, which were inhibited by U73122. Lastly, 18:2t10,c12 increased the abundance of PLCγ1 in the plasma membrane within 3 min. Taken together, these data suggest that PLC plays an important role in 18:2t10,c12-mediated activation of intracellular calcium accumulation, inflammatory signaling, delipidation, and insulin resistance in human primary adipocytes.

Introduction

Obesity is currently one of the most prevalent nutrition-related diseases in the US. The CDC estimated that 35.7% of U.S. adults were obese in 2010 (1). Consuming conjugated linoleic acid (CLA)-containing supplements and fortified foods, which were given Generally Recognized as Safe status by the FDA in 2008, has become a popular method for weight management. Within the last decade, at least 15 clinical studies (2, 3) and 30 animal (for review, see 4) studies showed that consuming an equal mixture of trans-10, cis-12 (18:2t10,c12) and cis-9, trans-11 (18:2c9,t11) CLA or 18:2t10,c12 alone reduced body fat or increased fat-free mass. 18:2c9,t11 and 18:2t10,c12 are the 2 major CLA isomers with known biological functions (4, 5). 18:2c9,t11 is the predominant CLA isomer in dairy products, whereas an equal mixture of 18:2c9,t11 and 18:2t10,c12 is found in most CLA-containing weight loss supplements and fortified foods. However, it is only the 18:2t10,c12 isomer that causes adipocyte delipidation (6, 7) and loss of body fat (8, 9).

The proposed anti-obesity mechanisms of 18:2t10,c12 include increasing energy expenditure, inhibiting adipogenesis and lipogenesis, stimulating lipolysis, and promoting adipocyte apoptosis (4). We previously demonstrated that 18:2t10,c12-mediated suppression of adipogenesis and lipogenesis and adipocyte delipidation were dependent on inflammatory signaling (7, 10, 11) and linked to endoplasmic reticulum (ER) release of calcium and increased intracellular calcium accumulation (12). Upregulation of inflammatory pathways by 18:2t10,c12 increased the activity of nuclear factor kappa B (NFκB), activator protein (AP)-1, and mitogen-activated protein kinases (MAPK) (7, 10, 11), which lead to inhibition of PPARγ abundance and activity (13–15), reduction of glucose and fatty acid uptake, and de novo lipogenesis (6, 7, 14). However, the upstream signals activated by 18:2t10,c12 that initiate this inflammatory signaling cascade are unclear (11).

Heat shock proteins (HSPs) exist ubiquitously in cellular organelles, including the cytoplasm, mitochondria, golgi, and nucleus (16). They respond to various stressors, including heat, pH changes, inflammation, oxidative injury, and viral agents. They have strong cytoprotective effects and function as molecular chaperones in protein folding, transport, and degradation (17). Relevant to this study, several HSPs (e.g., the HSPA family of HSP70s) are linked to intracellular calcium accumulation (18) and inflammatory signaling pathways, including NFκB, extracellular signal-related kinases (ERKs), and phospholipase c (PLC) (19–21). However, the role of HSPs in 18:2t10,c12-mediated inflammatory signaling is unknown.

We recently discovered that blocking diacyglycerol kinase (DGK) activity using the chemical compound R59022 or RNA interference targeting DGK attenuated 18:2t10,c12-induced intracellular calcium accumulation, inflammatory signaling, and

suppression of lipogenesis (22), suggesting a role for DGK in 18:2t10,c12-regulated cell signaling. Based on the well-known role of PLC as an upstream enzyme that produces diacylglycerol (DAG), a substrate for DGK, and inositol-3-phosphate (IP3), an inducer of calcium release from the ER, we hypothesized that PLC is an upstream activator of these cellular responses to 18:2t10,c12. To test this hypothesis, 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione (U73122), a universal PLC inhibitor, was used in primary cultures of human adipocytes treated with 18:2t10,c12. Data from this study suggest that PLC plays an important role in 18:2t10,c12-mediated activation of inflammatory signaling, insulin resistance, and delipidation.

Materials and Methods

Materials

All cell culture ware and Hyclone FBS were purchased from Fisher Scientific. Taqman assays were purchased from Applied Biosytems Inc, Foster City, CA. Lightning Chemiluminescence Substrate was purchased from Perkin Elmer Life Science. Immunoblotting buffers and precast gels were purchased from Invitrogen Life Technologies. Polyclonal antibodies for GAPDH, α-caveolin-1, and PLCδ4 and a monoclonal antibody for anti-PPARγ were obtained from Santa Cruz Biotechnology. Anti-total and anti-phospho (P) ERK, c-Jun-NH2-terminal kinase (JNK), cJun, and PLCγ1 antibodies were purchased from Cell Signaling Technologies. 18:2c9,t11 and 18:2t10,c12 (+98% pure) were purchased from Matreya. Fluo-3 acetyloxymethyl ester, pluronic F-127, and probenecid were purchased from Invitrogen by Life Technologies. Thapsigargin and ionomycin were purchased from Calbiochem-EMD Biosciences. The

PLC inhibitor, U73122, was purchased from Sigma Aldrich. Adipocyte media were purchased from Zen Bio. All other reagents and chemicals were purchased from Sigma Aldrich unless otherwise stated.

Culturing of human primary adipocytes

Abdominal white adipose tissue was obtained from Caucasian and African American females aged between 30 and 50 y with a BMI ≤32.0 during elective abdominoplasty with consent approved from the Institutional Review Board at University of North Carolina at Greensboro and the Moses Cone Memorial Hospital. Tissue was digested, stromal vascular cells were isolated, and cells were differentiated for 7–14 d as described (6, 7). Cells were pretreated with or without 5, 10, or 15 μmol/L of the PLC inhibitor U73122, followed by administration of BSA vehicle control, 18:2c9,t11, or 18:2t10,c12 for 10 min to 48 h, depending on the outcome measured. U73122 inhibits PLC by decreasing the activity of PLC's substrate phosphatidylinositol 4,5-bisphosphate (PIP2) (23). Each treatment was normalized to the same amount of BSA or ethanol vehicle control. Each independent experiment was repeated at least twice using a mixture of cells from 3 participants unless otherwise indicated.

Fatty acid preparation

18:2c9,t11 and 18:2t10,c12 were complexed to 7.5% fatty acid-free BSA (Sigma A7030, lot no. 040M1649) at a 4:1 molar ratio to make a 4-mmol/L stock concentration as previously described (7). 18:2c9,t11 and 18:2t10,c12 were given at physiological levels (50 μmol/L) unless otherwise indicated (24, 25).

RNA isolation and real-time qPCR

Total RNA was extracted, single-strand RNA was reverse-transcribed into complementary DNA, and real-time qPCR was performed as previously described (12).

3H-2-deoxy-D-glucose uptake

On d 10 of differentiation, serum-starved cultures were pretreated with or without U73122 for 30 min, followed by BSA vehicle control or 18:2t10,c12 for 48 h. Subsequently, 3H-2-deoxy-D-glucose uptake was measured as previously described (26).

Immunoblotting

Immunoblotting was conducted as previously described (7) using primary antibodies for P-ERK, total ERK, P-JNK, total JNK, P-cJun, total cJun, PLCδ4, PLCγ1, and caveolin-1 at 1:1000 dilutions followed by 1 h of exposure at room temperature to HRP-conjugated secondary antibodies at 1:5000 dilutions unless otherwise indicated. Primary and secondary antibodies targeting PPARγ were used at dilutions of 1:200 and 1:2000, respectively. Blots were exposed to chemiluminescence reagent for 1 min and X-ray films were developed using a SRX-101A Konica Minolta film developer.

Adipokine secretion in the media

Media were collected from the same set of cells that were used for immunoblotting. The supernatant from these cells was centrifuging at 13,200 × g for 10 min at 4°C to remove cell debris and kept at −80°C until analysis. The assay kits were obtained from Bio-Rad and the concentrations of adipocytokines were measured in the

media following the manufacturer's protocol using a BioPlex Suspension Array System (Bio-Rad).

Intracellular calcium concentrations

Intracellular calcium concentrations were measured using a calcium-sensitive fluorescent dye fluo-3 as previously described (12).

Plasma membrane fractionation

PLC isoforms acutely translocate to the cell membrane following activation and thus measuring their appearance in the membrane provides an indicator of activity. To determine if 18:2t10,c12 increased the activity of specific PLC isoforms, cells were treated with controls or 18:2t10,c12 and then the plasma membrane fractions were isolated and processed for immunoblotting using isoform-specific PLC antibodies. Briefly, cultures were changed to serum-free media 1 d prior to the experiment. On the day of the assay, cells were treated with BSA vehicle control, 18:2t10,c12, or thapsigargin, a positive control that induces intracellular calcium release, for 1, 3, or 6 min and immediately put on ice for processing. After washing with ice-cold HBSS twice, cells were gently scraped in 300 μL of Tris-buffered saline (pH 7.4) with 1% protease inhibitor containing 20 mmol/L Tris-HCl, 225 mmol/L sucrose, and 1 mmol/L EDTA and homogenized using a prechilled potter-elvahjem homogenizer system. Lysate was centrifuged at 16,000 × g for 20 min, supernatant was removed, and the pellet was resuspended in 500 μL Tris-buffered saline, rehomogenized, and subsequently layered onto a 1.2-mol/L sucrose cushion in a ultracentrifuge tube and centrifuged at 100,000 × g for 20 min as previously described (10). The suspended plasma membrane interphase

was collected using a syringe and pelleted after centrifuging at 100,000 × g for 30 min. The plasma membrane pellets were then resuspended in RIPA buffer containing protease inhibitor and kept on ice prior to determining the protein concentration for immunoblotting to detect PLC specific proteins and caviolin-1, a loading control.

Statistical analyses

Data for the dose-response studies were analyzed using 1-way ANOVA and Student's t test to compute individual pairwise comparisons of means ($P < 0.05$). Data for the time course x fatty acid treatment studies were analyzed by 2-way ANOVA testing the main effects of time (3, 6, 12, and 24 h) and fatty acid type (BSA, 18:2c9,t11, 18:2t10,c12) and their full-factorial interaction (time × fatty acid type). Tukey's multi-comparison test was conducted to detect treatment differences among the interactions ($P < 0.05$). All analyses were conducted on the JMP version 10.0 program (SAS). Data are expressed as means ± SEMs.

Results

The PLC inhibitor U73122 attenuates 18:2t10,c12-mediated insulin resistance and suppression of lipogenic protein or gene expression

We hypothesized that PLC was involved in 18:2t10,c12-mediated activation of inflammatory signaling and suppression of PPARγ activity and lipogenesis (7, 14) based on our published data showing that the PLC-phosphatidylcholine (PC)-specific inhibitor tricyclodecan-9-yl potassium xanthate (D609) attenuated markers of inflammation in 18:2t10,c12-treated adipocytes (12). Indeed, 50 μmol/L 18:2t10,c12 impaired insulin-stimulated glucose uptake within 48 h (Figure 3.1A), PPARγ protein abundance within 24

h (Figure 3.1B), and the mRNA levels of PPARγ and several of its target genes [e.g., insulin-dependent glucose transporter (GLUT)-4, acetyl CoA carboxylase (ACC)-1, and stearoyl-coenzyme A desaturase (SCD)-1] within 18 h of treatment (Fig. 1C). Consistent with our hypothesis, pretreatment with low amounts (i.e., 5–15 μmol/L) of the PLC inhibitor U73122 blocked or attenuated these antilipogenic effects of 18:2t10,c12.

U73122 inhibits 18:2t10,c12-mediated inflammatory signaling

To determine the extent to which 18:2t10,c12-mediated inflammatory signaling was dependent on PLC activation, primary human adipocytes were pretreated with U73122 for 30 min and then treated with 50 μmol/L 18:2t10,c12 for 18–24 h, and inflammatory gene and protein expression were measured. 18:2t10,c12-mediated induction of inflammatory genes [Figure 3.2A; Ca2+/calmodulin-dependent protein kinase (CaMK2)-β, cyclooxygenase (COX)-2, monocyte chemoattractant protein (MCP)-1, IL-6, and IL-8], secretion of inflammatory adipokines (Figure 3.2B; IL-6 and IL-8), and phosphorylation of ERK, JNK, and cJun (Figure 3.2C) were attenuated by pretreatment with micromolar amounts of U73122. Thus, U73122 inhibited 18:2t10,c12-mediated inflammatory signaling.

U73122 attenuates 18:2t10,c12-mediated induction of HSPs

18:2t10,c12 causes cell stress, including the integrated cell stress response (27) or ER stress (12, 28). Therefore, we wanted to determine the extent to which 18:2t10,c12 induced HSPs and if PLC was involved in this induction. Indeed, 50 μmol/L 18:2t10,c12 increased the mRNA levels of several HSPs, including HSPA1A, HSPA6, and HSPH1 as early as 6 h, with the greatest induction at 12 h of treatment (Figure

3.3A). Pretreatment with U73122 attenuated the induction of HSPs by 18:2t10,c12 (Figure 3.3B), suggesting that PLC is involved in 18:2t10,c12-mediated induction of several isoforms of HSPs associated with inflammatory signaling.

U73122 attenuates 18:2t10,c12-mediated intracellular calcium accumulation

The rapid increase in intracellular calcium accumulation caused by 18:2t10,c12 may serve as an initial inflammatory signal (12), because blocking calcium release from the ER prevented 18:2t10,c12-mediated inflammatory signaling and insulin resistance (5, 12). To determine the extent to which 18:2t10,c12-mediated increase in intracellular calcium was dependent on PLC signaling, adipocytes were pretreated with U73122 for 30 min and then treated with 18:2t10,c12 for up to 10 min and intracellular calcium concentrations were measured. As expected, 18:2t10,c12 increased the accumulation of intracellular calcium within several minutes, reaching a peak after 3 min (Figure 3.4). Notably, this increase of intracellular calcium was attenuated by pretreating the cells with low amounts of U73122 (Figure 3.4), suggesting that PLC is involved in 18:2t10,c12-mediated intracellular calcium accumulation.

18:2t10,c12 increases mRNA levels of PLCδ4 and the translocation of PLCγ1 protein

Fifty micromolars of 18:2t10,c12, but not 18:2c9,t11, induced the mRNA levels of PLCδ4 after 12 h of treatment (Figure 3.5A) and this induction was inhibited in a dose-dependent manner by U73122 (Figure 3.5B). However, 18:2t10,c12 did not acutely increase the translocation of PLCδ4 to the plasma membrane (Figure 3.5C). In contrast, 18:2t10,c12 increased the translocation of PLCγ1 protein to the plasma membrane within 3 min (Figure 3.5C) but did not induce the mRNA levels of PLCγ1 (Figure. 3.5A,B).

Collectively, these data suggest that 18:2t10,c12 increases the activity of 1 (i.e., PLCγ) of the 3 (i.e., PLCγ, PLCδ, PLCβ) major PLC isoforms found in human adipose tissue. However, gene silencing studies targeting these candidate isoforms of PLC are needed to confirm these findings, given the lack of specificity of many chemical inhibitors.

Discussion

Based on data presented in this article along with our previously published work on 18:2t10,c12, we proposed the following working model (Figure 3.6). We postulate that 18:2t10,c12 activates within seconds the specific cell surface receptors, including G protein coupled receptor (GPCR), G protein receptor (GPR), or protein tyrosine kinases. Activation of these receptors stimulates the translocation of specific isoforms of PLC (i.e., PLCγ1) to the plasma membrane within 3 min, thereby generating DAG and IP3 from PIP2. DAG is rapidly converted to phosphatidic acid and, along with IP3, triggers calcium release from ER within 3 min. The 18:2t10,c12-mediated increase in intracellular calcium in turn: 1) increases the transcription of HSPs within 6–12 h; 2) upregulates the transcription of calcium-specific isoforms of PLC (e.g., PLCδ4) within 12 h; and 3) activates inflammatory signaling within 24 h. Inflammatory signaling subsequently antagonizes PPARγ abundance and activity within 24 h, thereby suppressing insulin-stimulated glucose uptake and lipogenesis within 48 h. These 18:2t10,c12-mediated events cause adipocyte delipidation. Taken together, these data suggest an important role of PLC in mediating inflammatory signaling and delipidation of adipocytes by 18:2t10,c12. However, isoform-specific, PLC knockdown studies are needed to validate this hypothesis.

HSPs and 18:2t10,c12

HSPs are molecular chaperones or stress-related proteins that help prevent or reverse protein misfolding, aggregation, and misassembly in response to cellular stressors (for review, see 29, 30). The main HSPs activated by stress include HSPA (i.e., HSP70s), HSPB (i.e., small HSPs), HSPC (i.e., HSP90s), and HSPH (i.e., HSP105/110) (for review, see 31). Relevant to the current study, the protein levels of HSP70 were decreased by U73122 in A431 epidermoid carcinoma cells, demonstrating their dependence on PLC activation (21). Furthermore, HSP70 has been shown to be activated by increased cytosolic Ca^{2+} concentrations (18). However, HSP70 has also been reported to have antiinflammatory actions, as demonstrated by inhibiting MAPK and NFκB pathways (19, 20; for review, see 32). HSPHs and HSPAs have been reported to suppress heat-induced stress as well (for review, see 33, 34). Consistent with these findings, our data suggest that 18:2t10,c12 induces cell stress as evidenced by increased concentrations of calcium within 3 min, several HSP family members within 6 h, inflammatory genes within 18 h, and insulin resistance within 48 h. 18:2t10,c12-mediated induction of HSPA1A, HSPA6, and HSPH1 were inhibited by U73122, suggesting that their induction was dependent on PLC.

PLC and 18:2t10,c12

PLC can be classified as PC or phosphoinositide (PI) specific, depending on its phospholipid substrate. PLC-PC is involved in cell proliferation, differentiation, apoptosis, inflammation, generation of reactive oxygen species, and hypoxia (for review, see, 35). Its specific inhibitor, D609, has been reported to decrease PC-PLC activity, possibly by chelation of Zn2+ on the active sites of the enzyme (35; for review, see 36). Consistent

with these data, we previously reported that D609 blocked a 18:2t10,c12-mediated increase in the concentrations of intracellular calcium and reactive oxygen species and the expression of IL-8, COX-2, activating transcription factor 3, and GADD34 (12). However, D609 did not prevent 18:2t10,c12-mediated suppression of adipogenic and lipogenic genes, e.g., PPARγ, GLUT4, and adipocyte fatty acid binding protein 4 (data not shown). Collectively, these data suggest that some, but not all of 18:2t10,c12's effects in adipocytes are dependent on PC-specific PLC.

Currently, 6 families and 13 isoforms of PI-specific PLC have been identified (for review, see 37). Most PLC isozymes are activated by GPCRs, protein tyrosine kinase, or both (38, 39; for review, see 40). Consistent with these data, we recently reported that 18:2t10,c12 increased the expression of GPR56 and GPCR5A, 2 tumor suppressor proteins found in inflamed tissues, and decreased the expression of GPR120, an antiinflammatory protein (41). Furthermore, 18:2t10,c12's induction of inflammatory genes was blocked by the GPR40/120 agonist GW9508 (41). In the current study, we examined 2 PLC isoforms based on the human UniGene database showing that PLCγ and PLCδ are 2 of the 3 adipose tissue-specific PI-PLC isozymes, PLCβ being the third (for review, see 42), and our microarray data suggesting that 18:2t10,c12 increased PLCδ4 expression (data not shown). PLCγ can be directly activated by protein tyrosine kinases or by several lipid-derived second messengers, including phosphatidic acid and arachidonic acid, in the absence of protein tyrosine kinase activation (40). In contrast, PLCδ can be activated by increased intracellular calcium concentrations (40) due to its calcium-binding C2 domain (43).

In the present study, 18:2t10,c12 increased intracellular calcium concentrations within 3 min of treatment, whereas it increased the expression of PLCδ4 after 12 h of

treatment but had no effect on PLCδ4 translocation. In contrast, 18:2t10,c12 had no effect on the expression of PLCγ1 but increased PLCγ1 translocation to the plasma membrane within 3 min. The time frame of this acute translocation of PLCγ1 to plasma membrane is consistent with work by Matsuda et al. (44). Notably, these 2 PLC isoforms have been linked to downstream inflammation. For example, overexpression of PLCδ4 has been reported to upregulate inflammatory ERK signaling in MCF-7 cells (45). Similarly, generation of DAG from PLCγ activates NFκB and MAPK signaling pathways (for review, see 46).

Interestingly, SFAs like palmitic and stearic acids also induce inflammatory signaling and insulin resistance in adipose tissue (for review, see 47). Consistent with these data, we previously demonstrated that stearic acid increased the mRNA levels of several proinflammatory genes in human adipocytes, which was further enhance by co-supplementation with 18:2t10,c12 (41). Although we showed that 18:2t10,c12 activates several inflammatory pathways shared by SFAs (for review, see 4, 47), including ERK, JNK, cJun, and NFκB (7, 10, 12, 22, 26, 41), we have not observed increases in the expression levels of toll-like receptor (TLR)4 or TLR2 in adipocytes treated with 18:2t10,c12 (data not shown). However, we have not chemically inhibited or silenced TLR4 or TRL2 to determine their role in 18:2t10,c12-mediated inflammatory signaling and insulin resistance. Currently, we speculate that 18:2t10,c12 activates inflammatory signaling via GPCR, GPR, or protein tyrosine kinase as shown in Figure 6.

Taken together, these data suggest that the rapid 18:2t10,c12-mediated increase in PLCγ1 is associated with the rapid increase of intracellular calcium, which triggers downstream inflammatory pathways that promote adipocyte delipidation. However, loss-of-function studies for PLCγ1 are needed to confirm this hypothesis. Alternatively, PLCβ,

another adipose tissue-specific PI-PLC isozyme, can also be activated by cell surface GPCR and warrants investigation.

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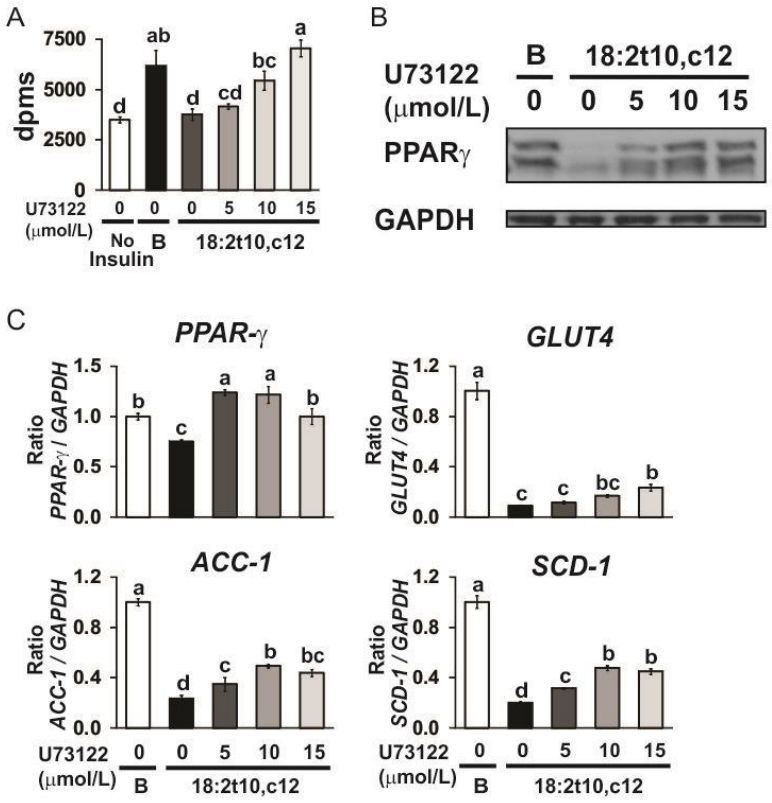

Figure 3.1 The PLC Inhibitor U73122 Attenuates 18:2t10,c12-Mediated Insulin Resistance and Suppression of Lipogenic Protein or Gene Expression in Human Adipocytes. (A) Human primary adipocytes were incubated in serum-free, low-glucose DMEM media for 24 h and pretreated with 5, 10, or 15 μmol/L U73122 for 30 min, followed by 50 μmol/L 18:2t10,c12 or BSA vehicle control (B) for 48 h. Insulin-stimulated glucose uptake was measured by scintillation radioactivity counting on the day of assay (n = 4/treatment). (B) Cultures were pretreated as in A, followed by 50 μmol/L 18:2t10,c12 or BSA vehicle control treatment for 24 h. The protein abundance of PPARγ was measured by immunoblotting (n = 3/treatment). (C) Cultures were pretreated as in A, followed by treatment of 50 μmol/L 18:2t10,c12 or BSA vehicle control for 18 h. The expression of PPARγ, GLUT4, ACC-1, and SCD-1 was measured by qPCR (n = 3–4/treatment). Means without a common letter

differ, $P < 0.05$. Data in A–C are representative of at least 3 independent experiments. ACC, acetyl-CoA carboxylase; GLUT4, insulin-dependent glucose transporter 4; PLC, phospholipase C; SCD, stearoyl-CoA desaturase; 18:2t10,c12, trans-10, cis-12; U73122, 1-[6-((17β-3-methoxyestra-1,3,5 (10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione.

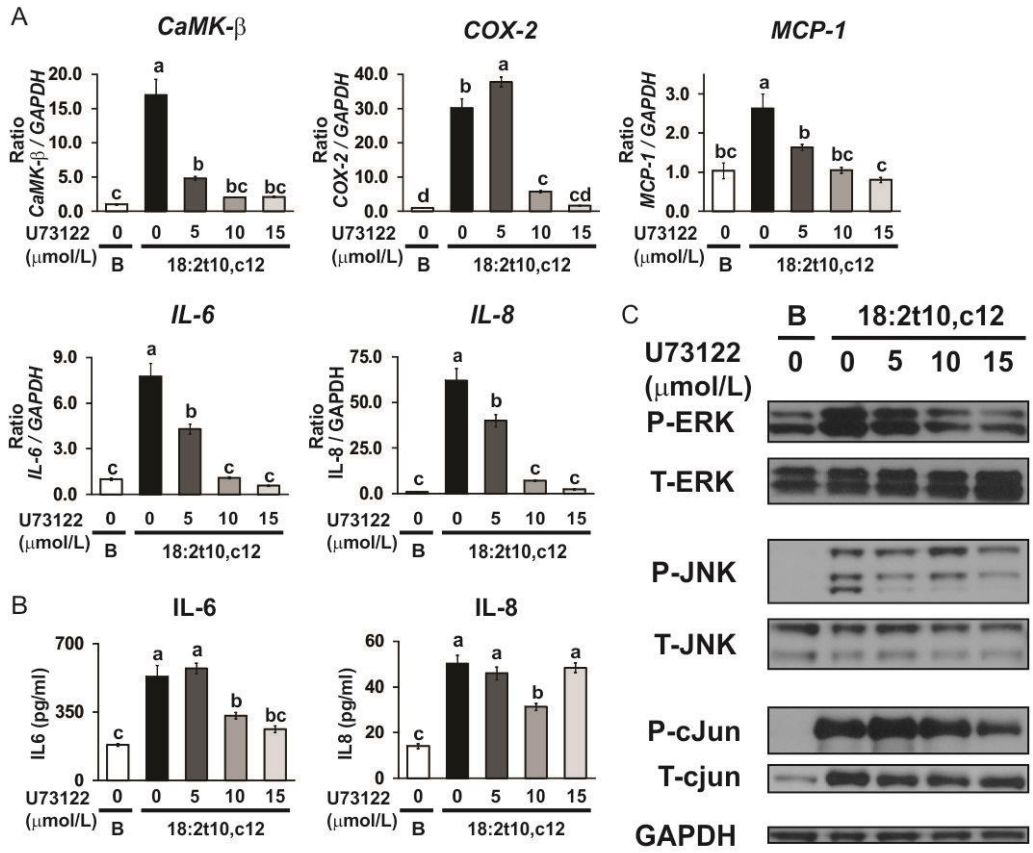

Figure 3.2 U73122 Inhibits 18:2t10,c12-Mediated Inflammatory Signaling in Human Adipocytes. (A) Human primary adipocytes were pretreated with 5, 10, or 15 μmol/L U73122 for 30 min, followed by 50 μmol/L 18:2t10,c12 or BSA vehicle control (B) for 18 h. Gene expression of CaMK2-β, COX-2, MCP-1, IL-6, and IL-8 was measured by qPCR (n = 3–4/treatment). (B) Cultures were pretreated as in A, followed by 50 μmol/L 18:2t10,c12 or BSA vehicle control treatment for 24 h. Media was collect to measure secreted proinflammatory markers (n = 3–4/treatment). (C) Cultures were used from B. Activation of inflammatory mediators, including ERK, JNK, c-Jun, and GAPDH, were measured by immunoblotting (n = 3–4/treatment). Means without a common letter differ, $P < 0.05$. Data in A–C are representative of at least 3 independent experiments. CaMK, Ca2+/calmodulin-

dependent protein kinase; COX, cyclooxygenase; ERK, extracellular signal-related kinase; JNK, c-Jun N-terminal kinase; MCP, monocyte chemoattractant protein; P, phosphorylated; T, total; 18:2t10,c12, trans-10, cis-12; U73122, 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione.

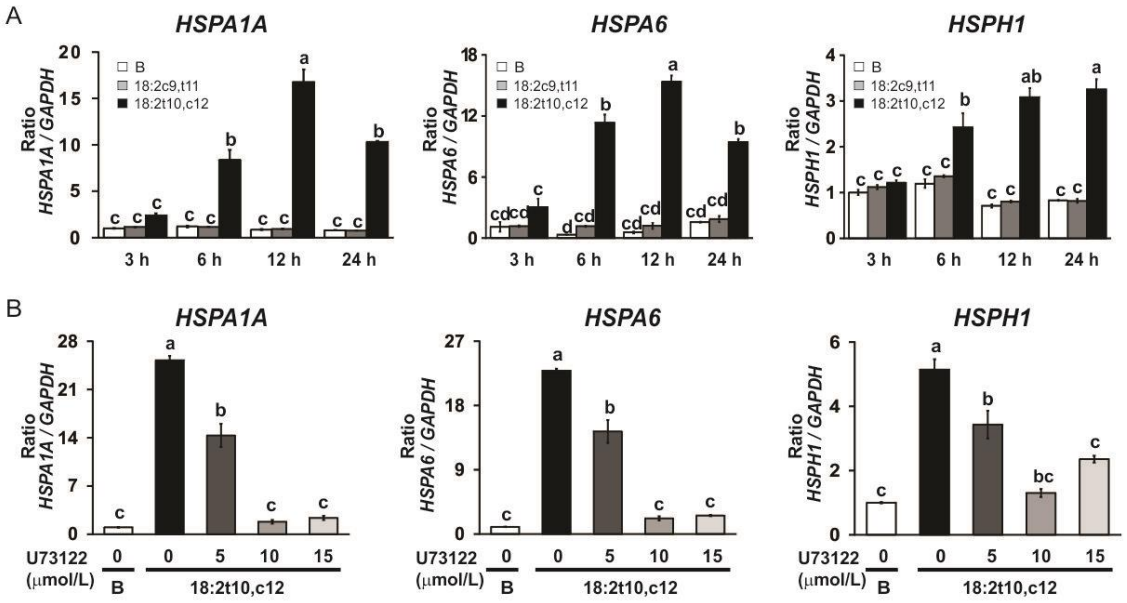

Figure 3.3 U73122 Attenuates 18:2t10,c12-Mediated Induction of HSPs in Hhuman Adipocytes. (A) Human primary adipocytes were treated with 50 μmol/L 18:2c9,t11, 18:2t10,c12, or BSA vehicle control (B) for 3, 6, 12, or 24 h. Gene expression of HSPA1A, HSPA6, and HSPH1 was measured by qPCR (n = 3–4/treatment). (B) Cultures were pretreated with 5, 10, or 15 μmol/L U73122 for 30 min, followed by treatment with 50 μmol/L 18:2t10,c12 or BSA vehicle control (B) for 12 h. Gene expression of HSPA1A, HSPA6, and HSPH1 were measure by qPCR (n = 3–4/treatment). Means without a common letter differ, $P < 0.05$. Data in A, B are representative of 2–3 independent experiments. 18:2c9,t11, cis-9, trans-11; HSP, heat shock protein; 18:2t10,c12, trans-10, cis-12; U73122, 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione.

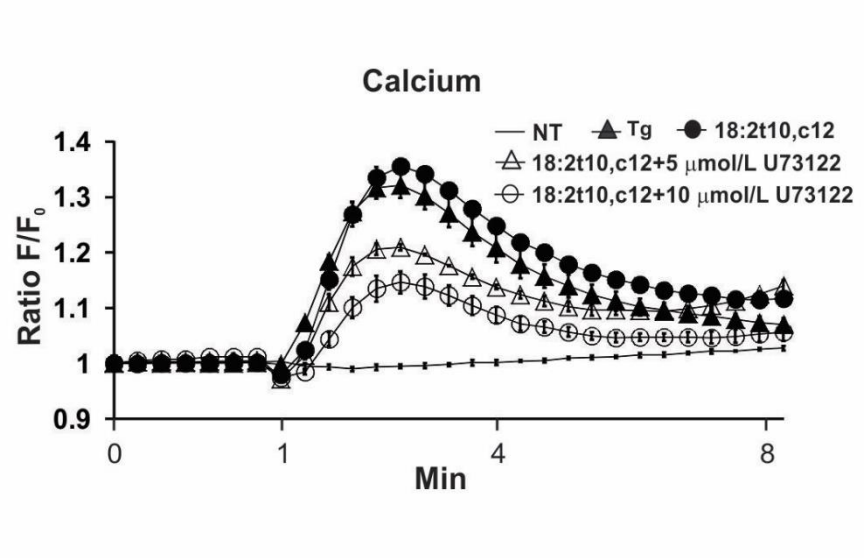

Figure 3.4 U73122 Attenuates 18:2t10,c12-Mediated Intracellular Calcium Accumulation in Human Adipocytes. Human primary adipocytes were incubated in 5 μmol/L Fluo-3 for 30 min, then treated with 5 or 10 μmol/L U73122 for 10 min and followed by injection of 150 μmol/L 18:2t10,c12, 5 μmol/L thapsigargin (Tg), a positive control for stimulating calcium release from the ER, or vehicle (NT). A kinetic intracellular calcium curve was generated by measuring the change in intensity of fluorescence over time. Data (n = 4–6/treatment) are representative of 3 independent experiments. ER, endoplasmic reticulum; F/Fo, changes in the ratio of calcium-dependent fluorescence to pre-stimulus background fluorescence. 18:2t10,c12, trans-10, cis-12; U73122, 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione.

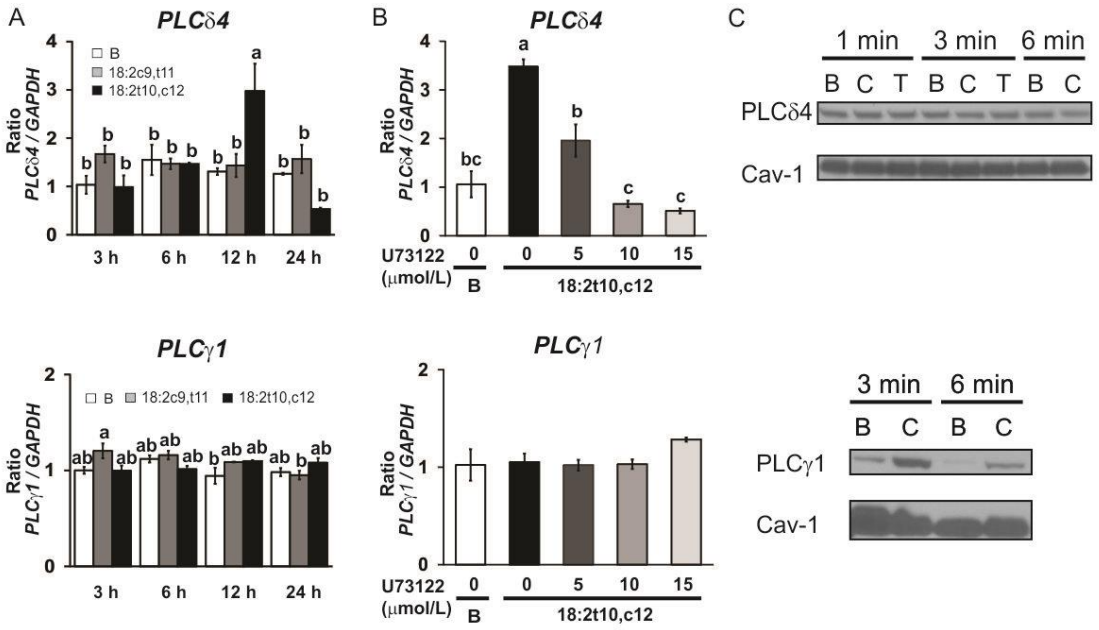

Figure 3.5 18:2t10,c12 Increases mRNA Levels of PLCδ4 and the Translocation of PLCγ1 Protein in Human Adipocytes. (A) Human primary adipocytes were treated with 50 μmol/L 18:2c9,t11, 18:2t10,c12, or BSA vehicle control (B) for 3, 6, 12, or 24 h. Gene expression of PLCδ4 and PLCγ1 was measured by qPCR (n = 3/treatment). (B) Another set of cells was pretreated with 5, 10, or 15 μmol/L U73122 for 30 min, followed by 50 μmol/L 18:2t10,c12 or BSA vehicle control (B). Gene expression of PLCδ4 and PLCγ1 were measured by qPCR (n = 3/treatment) (C). For measuring protein abundance, cultures were treated with vehicle control (B), 50 μmol/L 18:2t10,c12 (C), or 5 μmol/L thapsigargin (T) for 1, 3, or 6 min. Plasma membranes were then isolated and candidate PLC isomers were measured by immunoblotting (n = 3–4/treatment). Means without a common letter differ, $P < 0.05$. Data in A,B are representative of at least 2 independent experiments. Cav-1, caveolin-1; 18:2c9,t11, cis-9, trans-11; PLC, phospholipase C; 18:2t10,c12, trans-10,

cis-12; U73122, 1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione.

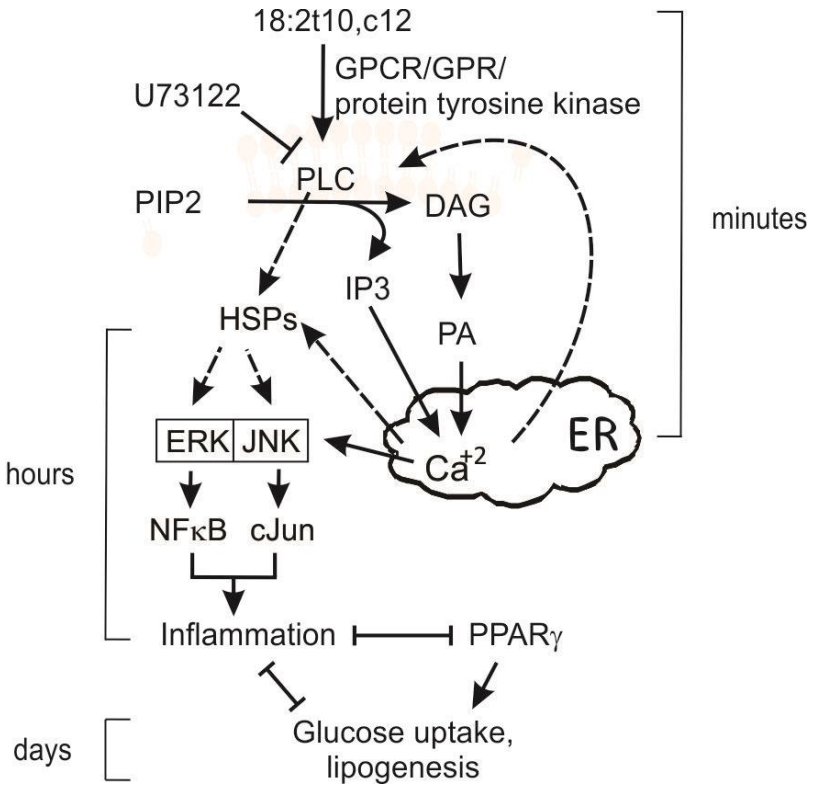

Figure 3.6 Working Model in Human Adipocytes. 18:2t10,c12 activates within seconds the specific cell surface receptors, including GPCRs, GPRs, or protein tyrosine kinases. Activation of these receptors stimulates the translocation of specific isoforms of PLC (i.e., PLCγ1) to the plasma membrane within 3 min, thereby generating DAG and IP3 from PIP2. DAG is rapidly converted to PA, and along with IP3, triggers calcium release from ER within 3 min. The 18:2t10,c12-mediated increase in intracellular calcium, in turn: 1) increases the transcription of HSPs within 6–12 h; 2) upregulates the transcription of calcium-specific isoforms of PLC (e.g., PLCδ4) within 12 h; and 3) activates inflammatory signaling within 24 h. Inflammatory signaling subsequently antagonizes PPARγ abundance and activity within 24 h, thereby suppressing insulin-stimulated glucose uptake and lipogenesis within 48 h. Together, these 18:2t10,c12-mediated events cause adipocyte delipidation. DAG, diacylglycerol; ER, endoplasmic reticulum; GPCR, G protein

coupled receptor; GPR, G protein receptor; IP3, inositol-3-phosphate; PA, phosphatidic acid; PIP2, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C.

CHAPTER IV

CONJUGATED LINOLEIC ACID REDUCES ADIPOSITY AND INCREASES MARKERS OF BROWNING AND INFLAMMATION IN WHITE ADIPOSE TISSUE OF MICE

Formatted for Shen W, Chuang CC, Martinez K, Reid T, Brown JM, Xi L,Hixson L, Hopkins R, Starnes J, McIntosh M. (2013). Conjugated linoleic acid reduces adiposity and increases markers of browning and inflammation in white adipose tissueof mice. *Journal of Lipid Research. 54(4),* 909-22. doi: 10.1194/jlr.M030924

Abstract

The objective of this study was to examine the mechanism by which conjugated linoleic acid (CLA) reduces body fat. Young male mice were fed three combinations of fatty acids at three doses (0.06%, 0.2%, and 0.6%, w/w) incorporated into AIN76 diets for 7 weeks. The types of fatty acids were linoleic acid (control), an equal mixture of trans-10, cis-12 (10,12) CLA plus linoleic acid, and an equal isomer mixture of 10,12 plus cis-9, trans-11 (9,11) CLA. Mice receiving the 0.2% and 0.6% dose of 10,12 CLA plus linoleic acid or the CLA isomer mixture had decreased white adipose tissue (WAT) and brown adipose tissue (BAT) mass and increased incorporation of CLA isomers in epididymal WAT and liver. Notably, in mice receiving 0.2% of both CLA treatments, the mRNA levels of genes associated with browning, including uncoupling protein 1 (UCP1), UCP1 protein levels, and cytochrome c oxidase activity, were increased in epididymal WAT. CLA-induced browning in WAT was accompanied by increases in mRNA levels of markers of inflammation. Muscle cytochrome c oxidase activity and BAT UCP1 protein levels were not affected by CLA treatment. These data suggest a linkage between decreased adiposity, browning in WAT, and low-grade inflammation due to consumption of 10,12 CLA.

Introduction

Obesity is recognized as the most widespread nutritional disease in the United States (1). Excess body fat increases chronic disease risk and mortality (2). Many Americans are consuming dietary supplements to lose weight or prevent weight gain, including supplements containing equal mixtures of cis-9, trans-11 (9,11) conjugated linoleic acid (CLA) and trans-10, cis-12 (10,12) CLA. Indeed, consuming a mixture of these CLA isomers, or 10,12 CLA alone, reduced body fat in many animal (reviewed in Ref. 3) and human studies (4). Rodents that consumed higher amounts of the CLA than humans (e.g., 0.5–1.5% CLA in the diet or 600–1,800 mg/kg body weight) lost body fat more rapidly but concurrently developed side effects, including chronic inflammation, insulin resistance, and lipoatrophy (5). Notably, intermediate levels of mixed CLA isomers (i.e., 0.1–0.3%, w/w) reduced adiposity in mice without adversely affecting liver weight or lipid content (6). However, individual isomers were not fed, the level of reduction in adiposity in the 0.1% group was marginal, and anti-obesity mechanisms were not identified (6). In contrast, clinical trials routinely use lower doses of CLA (e.g., 3–6 g/day or 35–70 mg/kg body weight), although the relative decrease in adiposity is not as rapid or great as in the higher doses used in rodent studies.

It has been reported that only the 10,12 CLA isomer reduces adiposity or delipidates adipocytes; however, at relatively high doses it also causes adverse side effects in mice (5) and some humans (7, 8). In contrast, the 9,11 CLA isomer has anti-inflammatory and adipogenic properties (9), and it improves insulin sensitivity in mice (10). Controversy still exists concerning the dose- and isomer-specific effects of CLA on adiposity, anti-obesity mechanism(s) of action, and adverse metabolic consequences. Continued existence of this controversy represents an important problem because, until

it is resolved, realizing the potential health benefits of taking CLA to decrease or prevent obesity and its adverse side effects will likely remain largely beyond reach.

Proposed anti-obesity mechanisms of 10,12 CLA include i) increased energy metabolism and expenditure, ii) decreased adipogenesis, iii) decreased lipogenesis and increased lipolysis, iv) inflammation, and v) adipocyte apoptosis (reviewed in Ref. 3). One possible linkage of these potential mechanisms to body fat loss, especially inflammation and energy expenditure, is browning of white adipose tissue (WAT). This would have important medical implications, because an increased brown fat-like signature is associated with lower risk of developing obesity in mice (11) and humans (12). We have shown that 10,12 CLA increases cyclooxygenase (COX)-2 expression and prostaglandin (PG) production in human adipocytes (13), which have been associated with induction of brown fat-like adipocytes in WAT (14, 15), suggesting a link between inflammation and browning. Consistent with this hypothesis, relatively high levels of an equal mixture of 10,12 and 9,11 CLA [1.5% (16)] or 10,12 CLA alone [0.5% (17); 1% (18)] increase markers of brown fat-like adipocytes, including uncoupling protein 1 (UCP1) in WAT in mice. However, these high levels of 10,12 CLA alone or mixed CLA isomers also cause adverse side effects as stated above.

Based on these data, we wanted to determine in mice i) the extent to which 10,12 CLA or a CLA isomer mixture given at low (i.e., an effective dose used in clinical trials), intermediate (i.e., 3.3-fold the effective clinical dose), and high doses (i.e., 10-fold the effective clinical dose) shifted free fatty acids (FFA) away from adipocyte storage and toward oxidation versus ectopic deposition; and ii) the role of WAT browning and inflammation in mediating the anti-obesity properties of CLA. In this study, we demonstrated that an intermediate dose of 10,12 CLA (i.e., 0.1% 10,12 CLA plus 0.1%

linoleic acid) or the CLA isomer mixture (i.e., 0.1% 10,12 CLA plus 0.1% 9,11 CLA) i) incorporated into WAT and liver, ii) decreased total WAT depot weight, iii) increased the mRNA levels of markers of browning, browning activators, and low-grade inflammation, and iv) increased the protein levels of UCP1, carnitine palmitoyltransferase (CPT)-1b, and COX-2 and the activity of cytochrome c oxidase in epididymal (EPI) WAT without decreasing food intake or causing steatosis or insulin resistance.

Methods

Experimental design and diets

129Sv male mice (n = 90) were obtained from Jackson Laboratories (Bar Harbor, ME) at 5–6 weeks of age and housed in pairs in a 12 h light/12 h dark cycle, temperature-controlled room. Ethical treatment of animals was assured by the UNCG Institutional Animal Care and Use Committee. After 1 week of acclimation to a standard rodent semipurified diet, mice were randomly assigned to one of nine dietary treatments (n = 10 mice per treatment; supplementary Table 4.1) for 7 weeks in this 3 × 3 factorial design (i.e., three levels of fatty acids and three types of fatty acids). The types of fatty acids used were linoleic acid (#1024, control FA indicated as “L” in the figures and tables); 10,12 CLA (#1249); and 9,11 CLA (#1245) purchased from Matreya LLC (Pleasant Gap, PA) (supplementary Table 4.1). The three doses of fatty acids in the diet (w/w) were 0.06% (low), 0.2% (intermediate), and 0.6% (high), equivalent to 70, 240, and 700 mg/kg body weight, respectively, assuming a 25 g mouse consumes 3 g of food per day. The low-dose (0.06%) diets were supplemented with i) 0.06% linoleic acid, ii) 0.03% 10,12 CLA plus 0.03% linoleic acid, or iii) 0.03% 10,12 CLA plus 0.03% 9,11 CLA. The intermediate-dose (0.2%) and high-dose (0.6%) diets were formulated in a

similar manner. This design ensured that each of the two types of 10,12 CLA-containing diets (i.e., 10,12 CLA plus linoleic acid and 10,12 CLA plus 9,11 CLA) within a given dose had the same amount of 10,12 CLA, allowing for determining additive or synergistic effects of 9,11 CLA when combined with 10,12 CLA, as is found in commercial CLA preparations. The low dose of mixed CLA isomers was chosen based on a clinical trial by Raff et al. (19) using one of the higher doses of mixed CLA (i.e., 6.4 g of 40/40% 9,11 CLA plus 10,12 CLA) in healthy subjects (i.e., postmenopausal women with BMI < 35.0) weighing on average 71 kg; equivalent to 77 mg of total CLA isomers or 62 mg 9,11 plus 10,12 CLA isomers/kg body weight. Total body fat mass was decreased in this mixed CLA group compared with controls after 16 weeks (19).

Treatments were added to a standard, semipurified rodent diet (D12450B; AIN76 containing 10% kcal from fat and 35% from sucrose) by Research Diets Inc., (New Brunswick, NJ). Diets were pelleted and packed under inert gas in individual 2.5 kg foil bags and stored at −20°C until use. The actual fatty acid profile of these diets is shown in supplementary Table 4.3. Fresh diet was provided twice per week to minimize oxidization. Mice had ad libitum access to both food and water. Food intake and body weight were measured weekly. Mice were euthanized by CO2 narcosis followed by cervical dislocation and exsanguination. Trunk blood was collected and serum was harvested for measuring markers of inflammation and lipoatrophy. WAT depots were harvested, weighed, frozen in liquid N2, and stored at −80°C until analysis.

Lipid extraction and fatty acid methylation

The dietary samples were ground into a powder with a coffee grinder (Black and Decker, Towson, MD). Total lipids from 1–2 g of the ground samples in triplicate were

extracted with 50 ml petroleum ether continuously for 4 h using a Glodfisch extraction apparatus (Labconco, Kansas City, MO). The liver and muscle tissue samples (100 mg) were homogenized in 0.9 ml water with a Sonic Dismembrator (550, Fisher Scientific, Pittsburgh, PA) for 30 s, and total lipids were subsequently extracted (20). The EPI WAT samples (30–50 mg) were used with no further processing. An internal standard containing 0.5 mg of heptadecanoic acid was then added to each lipid extraction and the EPI WAT. The fatty acids in the extracted lipids and the adipose tissues were methylated following alkali treatment. The fatty acids methyl esters were dissolved in 0.1–0.5 ml of hexane and analyzed using GC-MS.

GC-MS analysis

Fatty acid methyl esters were separated on a DB-23 capillary column (122-2332), 30 m × 0.25 mm, film thickness 0.25 μm (Agilent Technologies, Wilmington, DE) (21). Mass spectrometric analysis was conducted using a 6890 N model gas chromatograph (Agilent Technologies) equipped with an Agilent Technologies 5973N mass spectrometric detector. The oven temperature was programmed from 50°C to 100°C at 10°C/min, then to 200°C at 4°C/min, held for 2 min, and finally to 220°C at 4°C/min, held for 12 min. The average helium velocity was 36 cm/sec, and the split ratio was 100:1. The temperatures of the MSD electron ionization source and quadropoles were 230°C and 150°C, respectively. One microliter of methyl ester was manually injected, and the total fatty acid amounts were determined by the areas of the total ions for each fatty acid (Tables 4.1 and 4.2, supplementary Table 4.4).

Intraperitoneal glucose tolerance tests

Glucose tolerance tests (GTT) were performed during week 6 on nonanesthetized mice. Mice were deprived of food for 8 h and given an intraperitoneal glucose injection (Sigma-Aldrich, St. Louis, MO) at a dose of 1 g/kg body weight. Blood was obtained from the tail vein, and glucose levels were determined at 0, 5, 15, 30, 60, and 120 min following glucose administration using Contour blood glucose monitoring system (Bayer Diabetes Care, Tarrytown, NY). Total GTT area under the curve (AUC) was calculated as described (22).

Analysis of serum cytokine, chemokine, triglyceride, FFA, and insulin levels

Serum tumor necrosis factor (TNF) α, interleukin (IL)-6, IL-1β, and monocyte chemoattractant protein (MCP)-1 levels were determined using the Bio-Plex magnetic bead-multiplex immunoassay on the Bio-Plex 200 system, according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Serum triglyceride (TG) and FFA levels were determined using commercial assays from Thermo Scientific (Infinity TG assay #TR22421 and TG standards #TR22923, Norcross, GA) and Wako Diagnostics (#NEFA-HR-2, Richmond, VA), respectively. Serum insulin levels were detected using an ultrasensitive mouse insulin kit (Crystal Chem Inc., Downers Grove, IL). The homeostasis model assessment method (HOMA) for insulin resistance (IR) used the following formula: [fasting insulin concentration (ng/ml) × 24 × fasting glucose concentration (mg/dl)] / 405 (23).

Liver TG content and staining

Liver TG levels were measured by extracting lipids in 2:1 CHCl3/methanol in glass tubes at room temperature overnight. After centrifugation and reextraction, the

pooled lipid extract was dried down under N2 gas at 55°C and redissolved in a measured volume of 2:1 CHCl3/methanol. Dilute H2SO4 was added to the sample, which was then vortexed and centrifuged to split the phases. The aqueous upper phase was aspirated and discarded, and an aliquot of the bottom phase was removed and dried down; 1% triton-X100 in CHCl3 was then added, and the solvent was evaporated. Deionized water was added to each tube and vortexed until the solution was clear. Lipids were then quantified using the TG/GB kit (Roche). Next, the delipidated liver was dried down at 60°C for 1–2 h. Then, 1N NaOH was added and incubated at 60°C, with vortexing every 30 min to ensure complete tissue dissolution. Lowry protein assay was performed to determine liver protein concentration. For liver histological analyses, formalin-fixed, paraffin-embedded liver tissues were sliced and stained with hematoxylin and eosin.

Tissue RNA analysis and real-time quantitative PCR

Epididymal (EPI), inguinal (ING), and retroperitoneal (RET) WAT were harvested, and total RNA was extracted using RNeasy Lipid Tissue Kit (Qiagen, Valencia, CA) combined with RNase-Free DNase Set (Qiagen). EPI WAT was isolated from the bilateral intra-abdominal visceral depots attached to the epididymis. ING WAT was isolated from the bilateral superficial subcutaneous WAT depots between the skin and muscle fascia just anterior to the lower segment of the hind limbs. RET WAT was isolated from the bilateral visceral depots in the abdominal cavity behind the peritoneum on the dorsal side of the kidneys. Mesenteric WAT was isolated from a glue-like visceral net located in the mesentery of the intestines. RNA integrity was assessed using Agilent RNA 6000 Nano Kit on an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, CA). For real-

time qPCR, 1 μg total RNA from mouse tissues was converted into first-strand cDNA by using a high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). The qPCR was performed in a 7500 FAST real-time PCR system by using Taqman gene expression assays (Applied Biosystems). Fold differences in gene expression were calculated as 2−ΔΔCt using the endogenous reference gene TATA-binding protein (TBP).

Cytochrome c oxidase

Cytochrome c oxidase, the final protein complex in the mitochondrial electron transport chain, was used as an indicator of mitochondria content in tissue. Approximately 50 mg of the gastrocnemius-plantaris muscle group was homogenized on ice in a Potter Elvehjem homogenizer in 19 vol of 50 mM KH2PO4, 0.1 mM EDTA, and 0.1% Triton X-100 (pH 7.4), and then centrifuged at 10,000 g for 5 min at 4°C. Fat from the EPI depot was homogenized in 4 vol of 50 mM KH2PO4, 0.1 mM EDTA, and 0.25% Triton X-100 (pH 7.4). Enzyme activity in the muscle supernatant and fat homogenate was determined polarographically at 37°C with a Clark-type oxygen electrode as previously described (24).

Immunoblotting

Cellular protein was harvested in a phosphate buffered saline (pH = 7.5) lysis buffer containing 1% NP40, 0.5% SDS, 30 μl/ml aprotinin, 1 mM phenylmethylsulfonlyl fluoride, and 1 mM sodium orthovanadate. The samples were homogenized on ice and centrifuged at 15,000 g for 15 min at 4°C; then the protein concentration was determined in the supernatant using the bicinchoninic acid assay. Equal amounts of protein were

separated using 4–12% NuPage mini precast gels (Invitrogen Inc.), and transferred to PVDF membranes (Bio-Rad Inc.) as previously described (13). Membranes were then blocked with 5% milk in TBST for 30 min and washed with TBST 5 min three times. Overnight incubation of membranes at 4°C with primary antibodies for UCP1 (catalog no. ab23841, Abcam, Cambridge, MA); CPT-1b (catalog no. sc-20670, Santa Cruz Biotechnology Inc., Santa Cruz, CA); COX-2 (catalog no. sc-1745, Santa Cruz Biotechnology); and β-actin (catalog no. sc-1616, Santa Cruz Biotechnology) at 1:800, 1:400, 1:800, and 1:2,000, dilutions, respectively, followed by 1 h exposure at room temperature to horseradish peroxidase-conjugated secondary antibodies at 1:5000 dilutions unless otherwise indicated. Blots were exposed to chemiluminescence reagent for 1 min, and X-ray films were developed using a SRX-101A Konica Minolta film developer. Densitometry was performed on blots using the Kodak 4400 CF Image Station with the Kodak Molecular Imaging software.

Statistics

Statistical analyses were performed using a two-way ANOVA testing the main effects of fatty acid dose (0.06. 0.2, 0.6%) and fatty acid type (linoleic acid, 10,12 CLA, CLA isomer mixture) and their full-factorial interaction (dose × type) using the JMP version 8.0 program (SAS, Cary, NC), unless otherwise indicated. The significance levels of the main effects and interactions are shown in supplementary Tables V and VI. Tukey's multicomparison test was conducted to detect significant treatment differences among the interactions ($P < 0.05$). Data for cytochrome c oxidase were analyzed using a one-way ANOVA and Tukey's HSD test to compute individual pairwise comparisons of means ($P < 0.05$). Data are expressed as means ± SEM.

Results

CLA isomers incorporate into WAT and liver, but not muscle

To determine the extent to which CLA isomers incorporate into WAT, liver, and muscle fatty acids, we measured the fatty acid profile of these tissues. Indeed, 10,12 CLA and 9,11 CLA incorporated into EPI WAT (Table 4.1) and liver (Table 4.2) in a dose-dependent manner. Notably, mice supplemented with 10, 12 CLA or the CLA mixture had 5- to 20-fold higher concentrations of CLA isomers in EPI WAT compared with liver, demonstrating the accumulation in WAT and potential impact on cells comprising WAT. In contrast, CLA supplementation did not increase the CLA isomer content of muscle, with the exception of 9,11 CLA in the 0.2% dose of mixed CLA isomers (supplementary Table 4.4). There were no remarkable changes in the profiles of saturated, monounsaturated, or polyunsaturated fatty acid in WAT or muscle. In liver, however, the high dose of 10,12 CLA and the CLA mixture increased 5-fold the abundance of saturated and monounsaturated fatty acids.

Intermediate and high doses of CLA treatments decrease adiposity

The intermediate and high doses of both CLA treatments decreased total body weight gain (supplementary Table 4.5) and total WAT depot weights (e.g., adiposity index; Figure 4.1) compared with the linoleic acid (L) controls. Both CLA treatments decreased body and WAT depot weights to the same degree, with the EPI and ING depots losing the most grams of WAT. The abundance of several fatty acids in the EPI depot was decreased by the intermediate dose of both CLA treatments, including C10:0, C14:1, C16:1, and C18:3 n3 (Table 4.1). The weight of subscapular brown adipose tissue (BAT) was also decreased by intermediate and high doses of both CLA

treatments (Figure 4.1). Although food intake was not significantly ($P > 0.05$) reduced by CLA treatments, it was 4–7% lower in mice fed 10,12 CLA compared with mice fed linoleic acid alone. The high dose of both CLA treatments impaired the efficiency of food conversion to body weight gain compared with the linoleic acid controls (supplementary Table 4.5). Collectively, these data show that the reduction in adiposity by CLA is dose- and WAT depot-dependent and that the inclusion of 9,11 CLA with 10,12 CLA in the diets of mice does not further reduce adiposity.

High dose of CLA treatments cause hepatic steatosis, hepatomegaly, and elevated serum levels of MCP-1

Given the reported adverse side effects of high doses of CLA in rodents (reviewed in Refs. 3, 5), we examined the effects of three doses of CLA on these outcomes. The high dose, but not the low or intermediate doses, of both CLA treatments caused hepatic steatosis (Figure 4.2A), increased FA levels (data not shown), hepatomegaly (Figure 4.2B), and elevated serum levels of MCP-1 (Figure 4.3A), a proinflammatory chemokine that recruits macrophages to WAT. Serum levels of IL-6 were elevated in the intermediate dose of the CLA isomer mixture (Figure 4.3A). Notably, none of the CLA treatments increased serum levels of IL-1β, TNFα, or FFA (data not shown), nor increased TG levels (Figure 4.3B), nor impaired fasting glucose, insulin, or glucose tolerance (supplementary Table 4.5). These data suggest that there is a threshold dose (i.e., 0.1% 10,12 CLA plus 0.1% linoleic acid or 0.2% mixed CLA isomers) for both CLA treatments in mice that reduce adiposity without causing steatosis.

Intermediate or low doses of 10,12 CLA or mixed CLA isomers increase mRNA markers or activators of browning in a WAT depot-specific manner

To better understand how CLA reduces adiposity, we measured the mRNA levels of genes associated with browning and fatty acid oxidation in visceral (i.e., EPI, RET) and subcutaneous (i.e., ING) WAT. CLA treatments impacted multiple markers of browning in WAT in a depot-specific manner (Figure 4.4, supplementary Figure 4.1). For example, UCP1 (uncouples respiration from ATP synthesis), elongation of very long chain fatty acids 3 protein (Evol3; elongates fatty acids), cell death-induced DNA fragmentation factor-a-like effector A (Cidea; regulates lipid droplet formation), CPT-1b (facilitates fatty acid transport into mitochondria for oxidation), cytochrome c oxidase VIII b (Cox8b; promotes electron transfer during mitochondrial respiration), and peroxisome proliferator-activated receptor α (PPARα induces genes associated with fatty acid oxidation) were increased approximately 1- to 100-fold by the intermediate dose of 10,12 CLA or the CLA isomer mixture in EPI WAT (Figure 4.4A1). In RET WAT, the intermediate dose of both CLA treatments increased the expression of CPT-1b, Cox8b, and PPARα (supplementary Figure 4.1A). In contrast, only the low dose of the CLA isomer mixture increased these markers of browning in ING WAT (Figure 4.4B1). Interestingly, the mRNA levels of transmembrane protein (TMEM) 26, a gene expressed in 129Sv mice and human beige adipocytes, which are distinct from white or brown adipocytes and inducible with cAMP analogs or cold exposure (25), were increased by the high dose or the intermediate and high dose of 10,12 CLA in the EPI and ING depots, respectively. Unexpectedly, the high doses of both CLA treatments did not increase markers of browning in EPI, RET, or ING WAT, with the exception of CPT-1b in

EPI WAT, even though these CLA-treated mice had markedly decreased adiposity and impaired food conversion efficiencies, a hallmark of increased thermogenesis.

We have previously shown that 10,12 CLA increases the expression of COX-2, a nuclear factor kappa B (NF-κB)-inducible gene, and PG production in human adipocytes (13), which has been linked to induction of brown fat-like adipocytes in WAT (14, 15). Consistent with our hypothesis, the low and intermediate doses of CLA treatments increased the expression of COX-2 and PGF2α synthase in EPI WAT (Figure. 4.4A2), the depot where CLA had its greatest effects on markers of browning. In RET (supplementary Figure. 4.1B) and ING WAT (Figure 4.4B2), the low dose of the CLA isomer mixture increased COX-2 and PGF2α synthase expression, which is consistent with the induction of browning in these depots. The intermediate dose of both CLA treatments increased PGF2α synthase expression in all three WAT depots.

Intermediate dose of CLA treatments increase the protein or activity levels of browning markers in WAT

Consistent with the mRNA data above, 0.1% 10,12 CLA plus 0.1% linoleic acid (10) and 0.2% CLA isomer mixture (M) increased the protein levels of UCP1, CPT-1b, and COX-2 and the activity of cytochrome c oxidase in EPI WAT compared with the linoleic acid (L) controls (Figure 4.5). In contrast, UCP1 protein levels in BAT and cytochrome c oxidase activity in muscle were not affected by CLA treatment (data not shown). Taken together, these data suggest that the CLA-mediated reduction in adiposity is more likely due to increased mitochondria activity in WAT and not in BAT or muscle.

CLA treatments increase mRNA markers of low-grade inflammation in WAT

Increased NF-κB-driven inflammation has recently been shown to prevent obesity by increasing energy expenditure (26, 27). Furthermore, low-grade inflammation in WAT is characterized by recruitment of macrophages, including classically activated M1 macrophages and alternatively activated M2 macrophages. Here, we show that the mRNA levels of the proinflammatory M1 macrophage markers MCP-1, IL-6, F4/80, and TNFα were elevated in the intermediate and high dose of 10,12 CLA plus linoleic acid or CLA isomer mixture in EPI (Figure 4.6A1), RET (supplementary Figure 4.2A), and ING (Figure 4.6B1) WAT.

Given the role of alternatively activated, M2 macrophages in inflammation, including clearing apoptotic cells and necrotic tissue (reviewed in Ref. 28), we examined the influence of CLA treatments on the expression of three markers of the M2 macrophage phenotype [i.e., arginase 1, mannose receptor c1 (Mrc1 or CD206), and Clec10 (CD301)] (29). In EPI WAT, only the intermediate dose of the CLA isomer mixture increased arginase 1 expression, whereas the intermediate and high doses of the CLA isomer mixture increased Mrc1 and Clec10a expression (Figure 4.6A2). In RET WAT, only the high dose of the mixed CLA isomers increased arginase 1, Mrc1, and Clec10 expression (supplementary Figure 4.2B). In ING WAT, the high dose of both CLA treatments increased Mrc1 and Clec10 expression (Figure 4.6B2). In general, M2 macrophage markers were expressed to the greatest extent in WAT having the highest expression levels of M1 macrophage markers, except for arginase 1 in EPI WAT.

Impact of CLA treatment on mRNA markers of lipogenesis and lipolysis

Given the similar decrease in adiposity by both CLA treatments in the intermediate and high doses, we determined the extent to which this reduction correlated

with changes in the expression of genes associated with lipogenesis and lipolysis. Surprisingly, only the highest dose of 10,12 CLA or the CLA isomer mixture decreased the mRNA levels of PPARγ or several of its target genes (i.e., perilipin, FABP4) in EPI WAT (Figure 4.7A1), RET WAT (supplementary Figure 4.3A), and ING WAT (Figure 4.7B1). The mRNA levels of hormone sensitive lipase and adipose tissue TG lipase, enzymes that control lipolysis, were decreased by the high dose of both CLA treatments in EPI (Figure. 4.7A2), RET (supplementary Figure 4.3B), and ING (Figure 4.7B2). In general, the expression of genes associated with lipogenesis and lipolysis were not affected by the intermediate dose of CLA treatments.

FFA receptors GPR120 and GPR40 in WAT are differentially regulated by CLA

FFA activate FFA receptors, G-protein receptors (GPR), and G-protein-coupled receptors (GPCR), which influence glucose and lipid metabolism, intracellular calcium levels, and signaling pathways (30–35). GPR40 (35, 36) and GPR120 (30, 37) are activated by long-chain FFA, and their activation is associated with increased intracellular calcium levels and extracellular signal-related kinase (ERK) activation, similar to what we have shown in human adipocytes treated with 10,12 CLA (38). Although we were unable to measure the activities of GPR120 and GPR40, we determined the effects of CLA on their expression levels. The low dose of both CLA treatments increased the expression of GPR120, whereas the high dose decreased GPR120 expression in all three WAT depots (Figure. 4.8, supplementary Figure. 4.4). Given the anti-inflammatory properties of GPR120 (30), our finding of the induction of proinflammatory M1 markers in the high dose of CLA treatments is consistent with decreased GRP120 expression in WAT. Although the expression levels of GRP40 were

extremely low in WAT, the high dose of 10,12 CLA plus linoleic acid or the CLA isomer mixture induced the expression of GPR40 (Figure. 4.8, supplementary Figure 4.4), particularly in the ING depot (Figure 4.8B). Taken together, these data suggest that the expression levels of the FFA receptors GPR120 and GPR40 in WAT are differentially regulated by CLA isomers and may play a role in CLA-mediated downstream signaling in WAT.

Discussion

We conducted this study to resolve contradictions in the literature concerning the dose- and isomer-specific effects of CLA on i) incorporation in WAT, liver, and muscle; ii) adiposity; iii) steatosis, hyperlipidemia, and insulin resistance; and iv) browning and inflammation in WAT. We used the 10,12 CLA isomer because it is the main isomer reported to reduce adiposity (reviewed in Ref. 3) and the CLA isomer mixture because it is used commercially in supplements and fortified foods and in most human studies. We purchased relatively pure 10,12 CLA and 9,11 CLA isomers to avoid issues related to chemical manipulation and storage associated with commercially available supplements. Linoleic acid was used as a control fatty acid, because it contains the same number of carbons and carbon-carbon double bonds as CLA. We chose the 129Sv mouse model because of its capacity to induce thermogenesis when exposed to cold or β-3 agonists compared with the C57Bl6J mouse (39). Young, growing mice fed a normal mouse diet were used in order to examine the ability of CLA to prevent the accumulation of body fat. Thus, we did not examine the ability of CLA to treat adult mice that were already overweight or obese. A 7-week feeding period was used based on work by Parra et al. (6).

CLA isomers incorporate into WAT and decrease adiposity

We demonstrated that 10,12 and 9,11 CLA isomers incorporated into WAT (Table 4.1) and liver (Table 4.2) and that 9,11 CLA in the 0.2% dose of the mixed CLA treatment incorporated into muscle (supplementary Table 4.4), consistent with reports of incorporation into rodent liver (40, 41) or human WAT and muscle (42). WAT was the preferred storage organ for CLA isomers, consistent with work by Andreoli et al. (40). As demonstrated in many rodent studies, the high level (0.6%) of both CLA treatments robustly decreased adiposity in growing mice (Figure 4.1). This reduction was not due to decreased food intake (supplementary Table 4.5). Consistent with work by Parra et al. (6), who fed 0.1% and 0.3% (w/w) mixed CLA isomers to young C57BL6 mice consuming a low-fat diet for 35 days, 129Sv mice fed the intermediate dose of both CLA treatments (i.e., 0.1% 10,12 CLA plus 0.1% linoleic acid or 0.2% of mixed CLA isomers) had decreased adiposity. In general, the intermediate and high doses of 10,12 CLA (i.e., 0.1% and 0.3%, respectively) were equally effective as the CLA isomer mixture (i.e., 0.2 and 0.6%, respectively) in reducing adiposity, suggesting the 9,11 CLA isomer had no additive or synergistic effect on reducing body fat when combined with 10,12 CLA. In contrast, the low dose of both CLA treatments did not reduce adiposity. Thus, there appears to be a threshold dose of 10,12 CLA alone (i.e., 0.1%) and mixed CLA isomers (i.e., 0.2%) that significantly reduces adiposity in young growing mice over a 7-week feeding period without causing steatosis. Strikingly, increasing the 10,12 CLA dose by 3-fold (i.e., 0.1% to 0.3%) caused steatosis, demonstrating the small window of opportunity for fat loss without causing steatosis.

Increase in steatosis and markers of inflammation

Consistent with several studies using 0.5% or more 10,12 CLA or a CLA isomer mixture in the diet (reviewed in Ref. 3), the high dose of both CLA treatments increased steatosis, as demonstrated by overt appearance of lipid in the liver (Figure. 4.2A), increased liver weight and TG content (Figure 4.2B), and saturated and monounsaturated FA content (Table 4.2). In contrast, the low and intermediate doses of both CLA treatments did not cause steatosis (Figure 4.2), hepatomegaly (Figure 4.2B), or markedly affect the FA profile. This increase in steatosis and lack of fasting hyperglycemia at the 0.6% level of CLA suggest insulin resistance and could be due to increased insulin levels (36, 43, 44), given insulin's lipogenic actions. However, CLA did not increase serum insulin levels or cause insulin resistance (i.e., no increase in HOMA-IR score) in our study. In parallel with steatosis, serum levels of MCP-1 and IL-6 (Figure 4.3A) and ING WAT mRNA levels of MCP-1 (Figure 4.6B1) were highest in the high-CLA groups. These data show that a relatively high dose of 10,12 CLA (0.3%) and an equal CLA isomer mixture (0.6%), equivalent to 10 times one of the maximum doses consumed by humans when expressed per kilogram of body weight (19), causes lipoatrophy, inflammation, and steatosis.

Alternatively activated M2 macrophages attenuate inflammation and protect against metabolic diseases, including insulin resistance (reviewed in Ref. 45). We found that the high dose of CLA treatments increased the expression of several M2 markers in WAT (i.e., Mrc, Clec10; Figure 4.6), along with markers of classically activated M1 macrophages (i.e., TNFα, MCP-1, F4/80; Figure 4.6). Thus, the increase in M2 markers in WAT of mice in the high-CLA dose may be due to their role in clearance of apoptotic cells or necrotic tissue in inflamed WAT (reviewed in Refs. 28, 30).

Increase in browning of WAT

Energy expenditure is a function of basal metabolic rate (BMR), adaptive thermogenesis, and physical activity. CLA has been proposed to reduce adiposity by enhancing energy expenditure via increasing BMR or thermogenesis, thereby increasing fat oxidation (46–50). Enhanced thermogenesis is associated with an upregulation of UCP1, which facilitates proton transport across the inner mitochondrial membrane, thereby diverting energy from ATP synthesis to heat production. UCP1 is expressed primarily in brown adipocytes, but it is inducible in brite adipocytes (14, 15, 39, 51–54) and linked to thermogenesis, fatty acid oxidation, and decreased adiposity (reviewed in Ref. 55). Brite or beige adipocytes in WAT can arise from mesenchymal stem cells that express PRDM16 (52) upon stimulation of PGC-1α by PPARγ agonists like rosiglitazone (14, 39). Alternatively, they can (trans) differentiate from mature white adipocytes (39, 53, 54). Notably, activation of COX-2 (14, 15) is a key initiator of brite adipocyte recruitment. COX-2 produces PGs that enhance mitochondrial biogenesis and increase the uncoupling capacity when activated with adrenergics or cold exposure (53).

Several studies have shown that high doses of a mixture of CLA isomers (1.5%) (16) or 10,12 alone (1%) (17) decreased adiposity and increased UCP1 and CPT-1b expression in EPI or RET WAT, respectively, supporting the hypothesis that CLA decreases body fat, at least in part, by uncoupling mitochondria in WAT. Consistent with our hypothesis, the intermediate dose of 10,12 CLA or the CLA isomer mixture increased mRNA or protein markers of browning (i.e., UCP1, Cidea, Elovl3, CPT-1b, Cox8b, PPARα); fatty acid oxidation (i.e., PPARα, CPT-1b); cytochrome c oxidase activity; and PG production (i.e., COX-2, PGF2α synthase) in EPI WAT (Figures. 4.4,

4.5) without causing steatosis (Figure 4.2). Unexpectedly, mRNA markers of browning were not increased by the high dose of CLA treatments in WAT, even though this dose decreased adiposity the most. Indeed, LaRosa et al. (18) showed that the expression of UCP1 in 10,12 CLA-treated mice gradually increased after 2, 4, 7, and 10 days of treatment, and then decreased 70% by day 17. Thus, it is possible that the high dose of CLA treatment increased WAT browning acutely, causing the most rapid depletion of lipid in WAT (i.e., lipoatrophy). Subsequently, the depletion of WAT caused a down-regulation of browning and other genes associated with energy metabolism by the end of the 7-week study. Alternatively, the robust decrease in WAT by the high dose of CLA could be due to general dedifferentiation or adipocyte apoptosis (reviewed in Ref. 3).

Effects of CLA on GPR40 and GPR120

10,12 CLA, but not 9,11 CLA, has been shown to inhibit fatty acid transport and cAMP content in tumors and ING WAT by activating an inhibitory GPCR, which was blocked using pertussis-toxin, an inhibitor of heptahelical GPCR associated with CXCR1 and CXCR2 receptors that are coupled to calcium mobilization and ERK activation (56). Moreover, CLA activates GPR40 and increases intracellular calcium accumulation in pancreatic β cells, thereby stimulating insulin secretion (36). Previously, we showed that 10,12 CLA-mediated decrease in glucose and fatty acid uptake in primary adipocytes was prevented by pertussis-toxin, possibly via inhibition of MEK/ERK signaling (57). Furthermore, we demonstrated that 10,12 CLA's activation of inflammatory signaling and suppression of PPARγ and glucose uptake were dependent on increased intracellular calcium levels (38). Consistent with these data, we found in this study that the high dose of CLA treatments increased the expression of GPR40 in ING WAT (Figure 4.8B),

suggesting a potential role of this FFA receptor in mediating some of the proinflammatory effects of CLA in WAT. GPR40 inhibitor or silencing experiments are needed to test this hypothesis.

The expression of the long-chain FFA receptor GPR120, which has been shown to be activated by omega-3 fatty acids and to repress macrophage-mediated inflammation (30), was increased by the low dose of CLA treatments in EPI (Figure 4.8A), RET (supplementary Figure. 4.4), and ING (Figure 4.8B) WAT, and decreased by the high dose of CLA treatments. Consistent with these data, we found that a relatively high dose of 10,12 CLA, but not 9,11 CLA or oleic acid, decreased GPR120 expression in human adipocytes (58). Thus, it is tempting to speculate that CLA suppression of GPR120 contributes to the severe proinflammatory status of mice consuming the high dose of CLA.

In summary, our data showed in young 129Sv mice that i) CLA isomers incorporate into WAT and liver in a dose-dependent manner; ii) there is an intermediate, threshold dose of 10,12 CLA (i.e., 0.1% 10,12 CLA plus 0.1% linoleic acid) and mixed CLA isomers (i.e., 0.1% 10,12 CLA plus 0.1% 9,11 CLA) that reduces adiposity without causing steatosis or elevating serum FFA, TG, glucose, or insulin levels; iii) an equal isomer mixture of CLA reduced adiposity to the same extent as 10,12 CLA plus linoleic acid, demonstrating that the 9,11 isomer of CLA does not further reduce body fat; iv) an intermediate dose of CLA treatments increases mRNA and protein markers of browning and cytochrome c oxidase activity in EPI WAT but not in BAT or muscle; v) an intermediate or high dose of CLA treatments increase the expression of several markers of low-grade inflammation in WAT; and vi) a high dose of CLA causes lipoatrophy, leading to steatosis and marked inflammation. Future kinetic studies are needed to

examine how and when intermediate doses of CLA isomers impact exogenous and endogenous glucose and fatty acid utilization in WAT and liver over time and whether these isomers alone or in combination increase energy expenditure in vivo. A greater understanding of how important GPR120 and GPR40 are as mediators of CLA signaling is needed.

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Table 4.1 Fatty Acid Content of Epididymal WAT*

mg/g tissue	0.06% diet			0.2% diet		
	0.06% diet	10	M	L	10	M
C10:0	0.22 ± 0.03^{a}	0.18 ± 0.02^{a}	0.22 ± 0.02^{a}	0.21 ± 0.03^{a}	0.06 ± 0.01^{b}	0.06 ± 0.00^{b}
C12:0	1.3 ± 0.1^{ab}	1.5 ± 0.2^{a}	1.8 ± 0.1^{a}	1.5 ± 0.2^{a}	0.8 ± 0.1^{b}	0.8 ± 0.0^{b}
C14:0	17.2 ± 0.6^{bc}	21.3 ± 0.4^{a}	19.7 ± 0.8^{ab}	17.1 ± 1.3^{bc}	15.0 ± 0.8^{c}	14.2 ± 0.4^{c}
C14:1	2.6 ± 0.2^{ab}	3.1 ± 0.1^{a}	2.8 ± 0.2^{ab}	2.4 ± 0.2^{b}	1.0 ± 0.1^{c}	1.1 ± 0.1^{c}
C15:0	1.5 ± 0.1	1.5 ± 0.0	1.5 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.3 ± 0.0
C16:0	141 ± 5	154 ± 2	144 ± 3	149 ± 9	153 ± 3	154 ± 3
C16:1	108 ± 4^{b}	139 ± 3^{a}	120 ± 5^{ab}	107 ± 7^{b}	82 ± 4^{c}	81 ± 4^{c}
C17:1	2.4 ± 0.1^{bc}	3.0 ± 0.0^{a}	2.8 ± 0.1^{ab}	2.4 ± 0.1^{c}	2.4 ±0.1^{c}	2.6 ± 0.1^{bc}
C18:0	7.2 ± 0.4	5.7 ± 0.2	6.0 ± 0.3	7.0 ± 0.5	5.1 ± 0.1	5.2 ± 0.1
C18:1c	200 ± 6	244 ± 3	227 ± 5	202 ± 10	243 ± 6	244 ± 3
C18:2c	168 ± 4^{b}	195 ± 7^{a}	184 ± 6^{ab}	177 ± 8^{ab}	173 ± 6^{ab}	160 ± 4^{b}
C18:2t	1.00 ± 0.17	1.00 ± 0.06	0.91 ± 0.03	0.96 ± 0.11	0.91 ± 0.07	0.97 ± 0.05
9,11 CLA	**1.14 ± 0.05^{c}**	**1.25 ± 0.04^{c}**	**3.14 ± 0.30^{b}**	**1.10 ± 0.05^{c}**	**1.06 ± 0.12^{c}**	**7.52 ± 0.14^{a}**
10,12 CLA	**0.22 ± 0.06^{c}**	**0.92 ± 0.04^{b}**	**1.00 ± 0.07^{b}**	**0.27 ± 0.03^{c}**	**2.32 ± 0.21^{a}**	**2.39 ± 0.06^{a}**
C18:3n3	16.7 ± 0.7^{a}	16.3 ± 0.6^{a}	14.8 ± 0.8^{a}	16.1 ± 0.9^{a}	8.8 ± 0.5^{b}	8.6 ± 0.3^{b}
C18:3n6	1.03 ± 0.08	0.98 ± 0.05	0.92 ± 0.07	0.88 ± 0.07	1.07 ± 0.08	1.02 ± 0.06
C20:0	0.25 ± 0.02	0.21 ± 0.01	0.24 ± 0.01	0.20 ± 0.02	0.17 ± 0.02	0.20 ± 0.01
C20:1	2.31 ± 0.11	2.25 ± 0.1	2.35 ± 0.13	2.42 ± 0.2	2.38 ± 0.1	2.47 ± 0.08
C20:2	1.35 ± 0.11	0.81 ± 0.02	0.82 ± 0.04	1.29 ± 0.09	0.59 ± 0.08	0.67 ± 0.01
C20:3n6	0.48 ± 0.04	0.21 ± 0.01	0.21 ± 0.01	0.46 ± 0.04	0.20 ± 0.01	0.19 ± 0.01
C20:4n6	0.85 ± 0.08	0.52 ± 0.02	0.54 ± 0.04	0.92 ± 0.05	0.74 ± 0.04	0.71 ± 0.03
SAF	169 ± 6	179 ± 7	182 ± 6	178 ± 11	176 ± 5	174 ± 3
MUFA	315 ± 9	377 ± 15	355 ± 10	316 ± 17	330 ± 8	332 ± 6
PUFA	191 ± 5^{ab}	217 ± 8^{a}	206 ± 7^{ab}	198 ± 8^{ab}	189 ± 6^{ab}	182 ± 4^{b}

*Means ± SEM, not sharing a common letter within the same row differ ($P < 0.05$). Data not available for 0.6% diet analysis due to insufficient amounts of epididymal WAT. L, linoleic acid control; 10, 10,12 CLA plus linoleic acid; M, 10,12 CLA plus 9,11 CLA. Bolded data represent detected amounts of 9,11 CLA and 10,12 CLA.

Table 4.2 Fatty Acid Content of Liver*

mg/g	0.06% diet			0.2% diet			0.6% diet		
tissue	L	10	M	L	10	M	L	10	M
C12:0	0.02 ± 0.00^{c}	0.02 ± 0.00^{c}	0.02 ± 0.00^{c}	0.04 ± 0.01^{bc}	0.05 ± 0.01^{bc}	0.05 ± 0.02^{bc}	0.02 ± 0.00^{c}	0.07 ± 0.01^{ab}	0.10 ± 0.02^{a}
C14:0	0.50 ± 0.06^{bc}	0.66 ± 0.13^{bc}	0.49 ± 0.08^{bc}	0.83 ± 0.18^{bc}	1.16 ± 0.12^{bc}	1.23 ± 0.28^{b}	0.42 ± 0.07^{c}	2.19 ± 0.22^{a}	2.62 ± 0.31^{a}
C14:1	0.05 ± 0.01^{d}	0.08 ± 0.01^{bcd}	0.06 ± 0.01^{cd}	0.09 ± 0.02^{bcd}	0.12 ± 0.02^{abcd}	0.15 ± 0.05^{abc}	0.05 ± 0.01^{d}	0.16 ± 0.02^{ab}	0.2 ± 0.03^{a}
C15:0	0.07 ± 0.01^{d}	0.07 ± 0.01^{d}	0.06 ± 0.01^{d}	0.09 ± 0.01^{cd}	0.15 ± 0.01^{b}	0.14 ± 0.02^{bc}	0.06 ± 0.01^{d}	0.22 ± 0.02^{a}	0.25 ± 0.02^{a}
C16:0	15 ± 1^{bc}	16 ± 2^{bc}	13 ± 1^{c}	20 ± 3^{bc}	30 ± 2^{b}	25 ± 4^{bc}	12 ± 2^{c}	65 ± 5^{a}	77 ± 7^{a}
C16:1	3.5 ± 0.4^{b}	4.7 ± 0.8^{b}	3.5 ± 0.5^{b}	5.6 ± 1.1^{b}	8.1 ± 0.9^{b}	8.6 ± 2.0^{b}	3.0 ± 0.5^{b}	15.6 ± 2.2^{a}	18.8 ± 3.1^{a}
C17:1	0.10 ± 0.01^{d}	0.13 ± 0.02^{cd}	0.10 ± 0.01^{d}	0.16 ± 0.03^{bcd}	0.30 ± 0.03^{b}	0.26 ± 0.05^{bc}	0.09 ± 0.02^{d}	0.57 ± 0.05^{a}	0.66 ± 0.05^{a}
C18:0	2.6 ± 0.1^{bc}	1.8 ± 0.2^{d}	2.2 ± 0.1^{cd}	2.6 ± 0.1^{bc}	3.0 ± 0.1^{b}	2.0 ± 0.1^{cd}	2.2 ± 0.2^{cd}	4.1 ± 0.2^{a}	3.8 ± 0.3^{a}
C18:1c	18 ± 2^{bc}	20 ± 3^{bc}	15 ± 1^{c}	26 ± 4^{bc}	35 ± 3^{b}	30 ± 5^{bc}	15 ± 2^{c}	79 ± 6^{a}	95 ± 9^{a}
C18:2c	9 ± 1	10 ± 1	9 ± 1	12 ± 2	17 ± 1	14 ± 2	8 ± 1	13 ± 1	13 ± 1
C18:2t	0.04 ± 0.01^{b}	0.04 ± 0.01^{b}	0.02 ± 0.01^{b}	0.06 ± 0.02^{b}	0.08 ± 0.01^{b}	0.06 ± 0.02^{b}	0.02 ± 0.01^{b}	0.19 ± 0.02^{a}	0.26 ± 0.03^{a}
9,11 CLA	$\mathbf{0.07 \pm 0.01^{c}}$	$\mathbf{0.09 \pm 0.01^{c}}$	$\mathbf{0.15 \pm 0.02^{c}}$	$\mathbf{0.09 \pm 0.01^{c}}$	$\mathbf{0.14 \pm 0.01^{c}}$	$\mathbf{0.51 \pm 0.08^{b}}$	$\mathbf{0.07 \pm 0.01^{c}}$	$\mathbf{0.11 \pm 0.01^{c}}$	$\mathbf{1.63 \pm 0.09^{a}}$
10,12 CLA	$\mathbf{0.04 \pm 0.00^{c}}$	$\mathbf{0.06 \pm 0.01^{c}}$	$\mathbf{0.06 \pm 0.01^{c}}$	$\mathbf{0.04 \pm 0.00^{c}}$	$\mathbf{0.17 \pm 0.01^{b}}$	$\mathbf{0.13 \pm 0.02^{b}}$	$\mathbf{0.04 \pm 0.00^{c}}$	$\mathbf{0.35 \pm 0.03^{a}}$	$\mathbf{0.41 \pm 0.02^{a}}$
C18:3n3	0.43 ± 0.06	0.49 ± 0.09	0.41 ± 0.04	0.55 ± 0.11	0.74 ± 0.05	0.63 ± 0.12	0.29 ± 0.05	0.27 ± 0.03	0.3 ± 0.02
C18:3n6	0.17 ± 0.02	0.18 ± 0.03	0.15 ± 0.02	0.24 ± 0.06	0.33 ± 0.03	0.22 ± 0.05	0.14 ± 0.03	0.29 ± 0.02	0.29 ± 0.02
C20:0	0.07 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.09 ± 0.02	0.15 ± 0.02	0.08 ± 0.02	0.06 ± 0.01	0.12 ± 0.01	0.09 ± 0.01
C20:1	0.3 ± 0.04^{cd}	0.33 ± 0.06^{cd}	0.23 ± 0.02^{d}	0.35 ± 0.05^{cd}	0.53 ± 0.03^{bc}	0.33 ± 0.05^{cd}	0.26 ± 0.05^{d}	0.76 ± 0.07^{ab}	0.97 ± 0.13^{a}
C20:2	0.26 ± 0.03^{bc}	0.24 ± 0.03^{c}	0.22 ± 0.02^{c}	0.28 ± 0.03^{abc}	0.38 ± 0.03^{ab}	0.26 ± 0.03^{bc}	0.23 ± 0.04^{c}	0.29 ± 0.03^{abc}	0.40 ± 0.03^{a}
C20:3n3	0.03 ± 0.00	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.04 ± 0.00	0.02 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.01 ± 0.00
C20:3n6	0.38 ± 0.03^{abc}	0.28 ± 0.02^{cd}	0.36 ± 0.02^{bcd}	0.41 ± 0.03^{ab}	0.49 ± 0.01^{a}	0.34 ± 0.02^{bcd}	0.32 ± 0.05^{bcd}	0.30 ± 0.02^{bcd}	0.24 ± 0.02^{d}
C20:4n6	4.0 ± 0.2^{bcd}	2.8 ± 0.4^{d}	4.0 ± 0.2^{bcd}	4.4 ± 0.3^{abc}	5.2 ± 0.1^{ab}	4.1 ± 0.2^{bcd}	3.6 ± 0.5^{cd}	5.4 ± 0.2^{a}	4.2 ± 0.3^{abc}
C20:5n5	0.09 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.08 ± 0.02	0.05 ± 0.01	0.04 ± 0.00	0.05 ± 0.01
C22:0	0.02 ± 0.00^{b}	0.00 ± 0.00^{b}	0.01 ± 0.00^{b}	0.01 ± 0.01^{b}	0.02 ± 0.01^{b}	0.05 ± 0.01^{b}	0.01 ± 0.00^{b}	0.03 ± 0.01^{b}	0.18 ± 0.05^{a}
C22:1	0.07 ± 0.01^{a}	0.06 ± 0.01^{ab}	0.08 ± 0.00^{a}	0.10 ± 0.01^{a}	0.10 ± 0.01^{a}	0.07 ± 0.01^{a}	0.07 ± 0.01^{a}	0.05 ± 0.02^{ab}	0.02 ± 0.01^{b}
C22:5	0.31 ± 0.03^{ab}	0.24 ± 0.02^{c}	0.31 ± 0.03^{ab}	0.37 ± 0.06^{ab}	0.45 ± 0.02^{ab}	0.31 ± 0.05^{ab}	0.34 ± 0.05^{ab}	0.65 ± 0.05^{a}	0.41 ± 0.05^{ab}
C22:6n3	2.3 ± 0.2^{bc}	2.1 ± 0.4^{bc}	2.6 ± 0.2^{abc}	2.5 ± 0.2^{abc}	3.4 ± 0.1^{a}	2.4 ± 0.1^{abc}	2.0 ± 0.3^{c}	3.0 ± 0.2^{ab}	2.3 ± 0.2^{bc}
C24:0	0.08 ± 0.01^{b}	0.07 ± 0.01^{b}	0.09 ± 0.01^{b}	0.09 ± 0.02^{b}	0.14 ± 0.01^{a}	0.08 ± 0.01^{b}	0.05 ± 0.01^{b}	0.08 ± 0.01^{b}	0.05 ± 0.01^{b}
SAT	18 ± 2^{bc}	19 ± 3^{bc}	16 ± 1^{c}	24 ± 3^{bc}	34 ± 2^{b}	29 ± 4^{bc}	15 ± 2^{c}	72 ± 5^{a}	84 ± 8^{a}
MUFA	22 ± 2^{bc}	26 ± 4^{bc}	19 ± 2^{c}	33 ± 5^{bc}	44 ± 4^{b}	39 ± 7^{bc}	18 ± 3^{c}	96 ± 8^{a}	115 ± 11^{a}
PUFA	18 ± 1^{bc}	17 ± 2^{c}	17 ± 1^{bc}	21 ± 2^{abc}	28 ± 1^{a}	23 ± 2^{abc}	15 ± 2^{c}	24 ± 1^{ab}	23 ± 1^{abc}

*Means ± SEM, not sharing a common letter within the same row differ ($P < 0.05$). L, linoleic acid control; 10, 10,12 CLA plus linoleic acid; M, 10,12 CLA plus 9,11 CLA. Bolded data represent detected amounts of 9,11 CLA and 10,12 CLA.

Supplemental Table 4.1 Diet Formulations*

	0.06% diet			0.2% diet			0.6% diet		
Ingredients	L	10	M	L	10	M	L	10	M
	gm	gm	gm	gm	gm	gm	gm	gm	gm
Casein	189.6	189.6	189.6	189.6	189.6	189.6	189.6	189.6	189.6
L-Cystine	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8	2.8
Corn Starch	298.5	298.6	298.6	298.6	298.6	298.6	298.6	298.6	298.6
Maltodextrin 10	33.2	33.2	33.2	33.2	33.2	33.2	33.2	33.2	33.2
Sucrose	331.7	331.8	331.8	331.8	331.8	331.8	331.8	331.8	331.8
Cellulose	47.4	47.4	47.4	47.4	47.4	47.4	47.4	47.4	47.4
Soybean Oil	23.1	23.1	23.1	21.7	21.7	21.7	17.7	17.7	17.7
Lard	19.0	19.0	19.0	19.0	19.0	19.0	19.0	19.0	19.0
Linoleic acid	**0.6**	**0.3**	0	**2.0**	**1.0**	0	**6.0**	**3.0**	0
9,11 CLA	0	0	**0.3**	0	0	**1.0**	0	0	**3.0**
10,12 CLA	0	**0.3**	**0.3**	0	**1.0**	**1.0**	0	**3.0**	**3.0**
Mineral Mix S10026	9.5	9.5	9.5	9.5	9.5	9.5	9.5	9.5	9.5
DiCalcium Phosphate	12.3	12.3	12.3	12.3	12.3	12.3	12.3	12.3	12.3
Calcium Carbonate	5.2	5.2	5.2	5.2	5.2	5.2	5.2	5.2	5.2
Potassium Citrate, 1 H_2O	15.6	15.6	15.6	15.6	15.6	15.6	15.6	15.6	15.6
Vitamin Mix V10001	9.5	9.5	9.5	9.5	9.5	9.5	9.5	9.5	9.5
Choline Bitartrate	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9	1.9
Total	1000	1000	1000	1000	1000	1000	1000	1000	1000

*L, linoleic acid controls; 10, 10,12 CLA plus linoleic acid; M, 10,12 CLA plus 9,11 CLA.

Supplemental Table 4.2 Fat Composition of the Diet*

	0.06% diet			0.2% diet			0.6% diet		
Ingredients	L	10	M	L	10	M	L	10	M
	%	%	%	%	%	%	%	%	%
Soybean Oil	54.1	54.1	54.1	50.8	50.8	50.8	41.4	41.4	41.4
Lard	44.5	44.5	44.5	44.5	44.5	44.5	44.5	44.5	44.5
Linoleic acid	**1.4**	**0.7**	0	**4.7**	**2.35**	0	**14.1**	**7.05**	0
9,11 CLA	0	0	**0.7**	0	0	**2.35**	0	0	**7.05**
10,12 CLA	0	**0.7**	**0.7**	0	**2.35**	**2.35**	0	**7.05**	**7.05**

*Data were expressed as a percentage of total fat. L, linoleic acid controls; 10, 10,12 CLA plus linoleic acid; M, 10,12 CLA plus 9,11 CLA.

Supplemental Table 4.3 Fatty Acid Analysis of the Diets *,**

	0.06% diet			0.2% diet			0.6% diet		
	L	10	M	L	10	M	L	10	M
C14:0	0.027	0.033	0.017	0.027	0.033	0.027	0.028	0.023	0.024
C16:0	0.509	0.607	0.359	0.525	0.605	0.502	0.460	0.468	0.462
C16:1	0.032	0.038	0.021	0.033	0.038	0.032	0.033	0.029	0.029
C18:0	0.171	0.226	0.157	0.201	0.231	0.188	0.152	0.174	0.188
C18:1	0.800	0.987	0.606	0.858	0.972	0.827	0.704	0.766	0.746
C18:2	1.521	1.822	1.023	1.652	1.787	1.515	1.685	1.452	1.228
C18:3 (n6)	0.004	0.006	0.005	0.004	0.006	0.005	0.004	0.004	0.004
C18:3 (n3)	0.198	0.243	0.137	0.188	0.232	0.206	0.158	0.136	0.150
9,11 CLA	**0.005**	**0.005**	**0.016**	**0.008**	**0.006**	**0.069**	**0.005**	**0.013**	**0.225**
10,12 CLA	**0.003**	**0.022**	**0.015**	**0.006**	**0.069**	**0.067**	**0.004**	**0.220**	**0.220**
C20:0	0.003	0.005	0.004	0.004	0.006	0.005	0.003	0.003	0.004
C20:1	0.007	0.010	0.008	0.009	0.011	0.009	0.007	0.008	0.009
C20:2	0.010	0.013	0.009	0.011	0.014	0.011	0.009	0.011	0.011
Fat%	3.369	4.096	2.410	3.637	4.069	3.517	3.309	3.300	3.364

* Data were expressed as a percentage of total fat. L, linoleic acid controls; 10, 10,12 CLA plus linoleic acid; M, 10,12 CLA plus 9,11 CLA.

**C20:3 (n6), C20:3 (n3), C20:4, C20:5, C22:0, C22:1, C22:2, C22:3, C22:4, C22:6, and C24:1 were underdectable.

Supplemental Table 4.4 Fatty Acid Content of Muscle*

mg/g	0.06% diet			0.2% diet			0.6% diet		
tissue	L	10	M	L	10	M	L	10	M
C12:0	0.03 ± 0.01	0.01 ± 0.00	0.02 ± 0.00	0.04 ± 0.02	0.02 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
C14:0	0.63 ± 0.14^{a}	0.30 ± 0.02^{abc}	0.36 ± 0.04^{abc}	0.63 ± 0.19^{a}	0.48 ± 0.07^{ab}	0.67 ± 0.07^{a}	0.33 ± 0.05^{abc}	0.07 ± 0.01^{c}	0.12 ± 0.04^{bc}
C14:1	0.18 ± 0.05	0.08 ± 0.01	0.11 ± 0.02	0.15 ± 0.03	0.13 ± 0.02	0.18 ± 0.02	0.09 ± 0.02	0.01 ± 0.00	0.01 ± 0.00
C15:0	0.05 ± 0.01	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.01	0.04 ± 0.01	0.05 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.02 ± 0.00
C16:0	6.0 ± 1.5^{a}	3.2 ± 0.2^{ab}	3.4 ± 0.3^{ab}	5.1 ± 0.9^{a}	3.8 ± 0.4^{ab}	4.7 ± 0.4^{ab}	3.4 ± 0.3^{ab}	1.6 ± 0.1^{b}	2.9 ± 1.1^{ab}
C16:1	5.1 ± 1.1	2.5 ± 0.2	3.1 ± 0.4	4.4 ± 0.8	3.8 ± 0.6	5.0 ± 0.5	2.8 ± 0.4	0.4 ± 0.1	0.8 ± 0.3
C17:1	0.08 ± 0.01	0.04 ± 0.00	0.05 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.05 ± 0.01	0.01 ± 0.00	0.03 ± 0.01
C18:0	0.77 ± 0.23	0.8 ± 0.04	0.60 ± 0.03	0.68 ± 0.04	0.56 ± 0.05	0.59 ± 0.03	0.67 ± 0.03	0.54 ± 0.02	0.56 ± 0.09
C18:1c	8.1 ± 2.0	3.8 ± 0.3	4.5 ± 0.5	5.5 ± 0.7	5.4 ± 0.7	6.8 ± 0.8	4.3 ± 0.5	1.5 ± 0.1	3.5 ± 1.5
C18:2c	4.5 ± 1.1	2.0 ± 0.2	2.4 ± 0.3	3.1 ± 0.5	2.8 ± 0.4	3.6 ± 0.4	2.5 ± 0.4	0.7 ± 0.1	0.9 ± 0.2
C18:2t	0.03 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.01	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.02 ± 0.01
9,11 CLA	**0.07 ± 0.01^{b}**	**0.03 ± 0.00^{b}**	**0.05 ± 0.01^{b}**	**0.05 ± 0.01^{b}**	**0.05 ± 0.01^{b}**	**0.14 ± 0.03^{a}**	**0.03 ± 0.00^{b}**	**0.01 ± 0.00^{b}**	**0.06 ± 0.02^{b}**
10,12 CLA	**0.04 ± 0.01^{b}**	**0.02 ± 0.00^{ab}**	**0.03 ± 0.00^{ab}**	**0.02 ± 0.00^{ab}**	**0.06 ± 0.01^{a}**	**0.06 ± 0.01^{a}**	**0.02 ± 0.00^{ab}**	**0.03 ± 0.00^{ab}**	**0.04 ± 0.01^{b}**
C18:3n3	0.33 ± 0.09	0.12 ± 0.01	0.15 ± 0.02	0.28 ± 0.09	0.17 ± 0.03	0.21 ± 0.03	0.13 ± 0.03	0.03 ± 0.01	0.04 ± 0.01
C18:3n6	0.08 ± 0.02	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.01	0.06 ± 0.02	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.06 ± 0.01
C20:1	0.07 ± 0.02	0.04 ± 0.00	0.04 ± 0.00	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.04 ± 0.00	0.03 ± 0.00	0.05 ± 0.02
C20:2	0.08 ± 0.03	0.04 ± 0.00	0.04 ± 0.00	0.05 ± 0.01	0.09 ± 0.04	0.05 ± 0.00	0.04 ± 0.00	0.03 ± 0.01	0.04 ± 0.01
C20:3n6	0.08 ± 0.05	0.05 ± 0.00	0.03 ± 0.00	0.05 ± 0.00	0.04 ± 0.01	0.04 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.04 ± 0.01
C20:4n6	0.80 ± 0.46	0.57 ± 0.03	0.38 ± 0.03	0.62 ± 0.06	0.37 ± 0.04	0.43 ± 0.02	0.58 ± 0.06	0.39 ± 0.02	0.42 ± 0.06
C22:5	0.13 ± 0.03	0.17 ± 0.01	0.12 ± 0.01	0.14 ± 0.03	0.12 ± 0.02	0.14 ± 0.01	0.21 ± 0.02	0.17 ± 0.02	0.16 ± 0.03
C22:6n3	0.62 ± 0.26	0.54 ± 0.04	0.39 ± 0.05	0.65 ± 0.07	0.33 ± 0.05	0.42 ± 0.33	0.59 ± 0.08	0.31 ± 0.02	0.32 ± 0.05
C24:0	0.04 ± 0.01	0.06 ± 0.01	0.04 ± 0.00	0.06 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.04 ± 0.00	0.04 ± 0.01
SAT	7.5 ± 1.8	4.4 ± 0.2	4.5 ± 0.3	6.6 ± 1.1	5.0 ± 0.5	6.1 ± 0.5	4.5 ± 0.4	2.3 ± 0.1	3.6 ± 1.2
MUFA	13.4 ± 2.7	6.5 ± 0.5	7.9 ± 0.9	9.6 ± 1.2	9.4 ± 1.3	12.1 ± 1.3	7.2 ± 0.1	2.0 ± 0.2	4.4 ± 1.9
PUFA	6.8 ± 1.9	3.6 ± 0.3	3.6 ± 0.4	4.7 ± 0.6	4.1 ± 0.5	5.1 ± 0.5	4.2 ± 0.5	1.8 ± 0.1	2.1 ± 0.4

*Means ± SEM, not sharing a common letter within the same row differ ($P < 0.05$). L, linoleic acid control; 10, 10,12 CLA plus linoleic acid; M, 10,12 CLA plus 9,11 CLA. Bolded data represent detected amounts of 9,11 CLA and 10,12 CLA.

Supplemental Table 4.5 The Effects of CLA on Body Weight Gain (BWG), Food Intake (FI), Food Conversion Efficiency (FCE), Fasting Blood Glucose (BG), Glucose Tolerance Test (GTT) Area Under the Curve (AUC), Fasting Insulin, and HOMA-IR Index*

Diets	Total BWG (g)	Total FI (per cage, g)	FCE (per cage)	Fasting BG (mg/dL)	GTT AUC	Fasting insulin (ng/mL)	HOMA-IR index
Type	< 0.0001	0.0003	< 0.0001	0.3661	0.7136	0.7590	0.9649
Dose	< 0.0001	0.9844	< 0.0001	0.2580	0.5534	0.0640	0.0378
Interacti -on	< 0.0001	0.6104	0.0016	0.1085	0.5043	0.0412	0.0956
0.06 L	5.7± 0.1^{ab}	276 ± 3.0	25.1 ± 1.5^{b}	78.1 ± 3.8	23640 ± 808	0.51± 0.11^{ab}	2.35± 0.55
0.06 10	5.5± 0.1^{abc}	265 ± 3.9	24.2 ± 0.9^{b}	74.8± 2.6	23344 ± 919	0.39± 0.05^{b}	1.69± 0.21
0.06 M	5.0± 0.2^{abc}	269 ± 4.1	28.3 ± 2.4^{b}	73.4± 3.8	21418 ± 995	0.53± 0.09^{ab}	2.31± 0.48
0.2 L	6.2± 0.2^{a}	280 ± 2.4	22.8 ± 1.3^{b}	79.8± 2.1	23112 ± 1124	0.60± 0.11^{ab}	2.85± 0.54
0.2 10	3.9± 0.1^{cde}	261 ± 4.7	33.6 ± 1.4^{b}	75.6± 4.7	24082 ± 1000	0.44± 0.08^{ab}	1.96± 0.37
0.2 M	4.4± 0.1^{bcd}	270 ± 2.2	31.2 ± 1.6^{b}	78.5± 4.0	23230 ± 1084	0.47± 0.06^{ab}	2.22± 0.35
0.6 L	6.4± 0.1^{a}	282 ± 2.1	22.2 ± 0.8^{b}	74.6± 2.8	22193 ± 469	0.49± 0.09^{ab}	2.28± 0.50
0.6 10	2.8± 0.1^{de}	262 ± 2.5	52.1 ± 5.2^{a}	77.9± 4.8	22436 ± 1126	0.94± 0.21^{a}	4.20± 0.98
0.6 M	2.6± 0.1^{e}	264 ± 2.3	53.7 ± 4.7^{a}	89.5± 4.9	23194 ± 1350	0.60± 0.09^{ab}	3.18± 0.64

*Means ± SEM, not sharing a common letter within the same row differ ($P < 0.05$). L, linoleic acid control; 10, 10,12 CLA plus linoleic acid; M, 10,12 CLA plus 9,11 CLA. Bolded data represent detected amounts of 9,11 CLA and 10,12 CLA.

Supplemental Table 4.6 Probability Levels for the Main Effects of Fatty Acid Type and Dose and Their Interactions for the Experimental Outcomes.

Dependent variable	Type	Dose	Interaction
Fig 1			
EPI	< 0.0001	< 0.0001	< 0.0001
ING	< 0.0001	< 0.0001	< 0.0001
MEN	< 0.0001	< 0.0001	0.0007
RET	< 0.0001	< 0.0001	0.0002
Total WAT	< 0.0001	< 0.0001	< 0.0001
BAT	< 0.0001	< 0.0001	0.0002
Fig 2B			
Liver Weight	< 0.0001	< 0.0001	< 0.0001
Liver TG	< 0.0001	< 0.0001	< 0.0001
Fig 3			
MCP1	0.0142	< 0.0001	0.0044
IL6	< 0.0001	0.001	0.0019
Serum TG	< 0.0001	< 0.0001	< 0.0001
Fig 4A			
UCP1	< 0.0001	< 0.0001	< 0.0001
Elovl3	< 0.0001	0.0001	0.0706
Cidea	< 0.0001	< 0.0001	< 0.0001
CPT1b	< 0.0001	< 0.0001	< 0.0001
Cox8b	< 0.0001	< 0.0001	< 0.0001
PPARα	< 0.0001	< 0.0001	< 0.0001
COX2	< 0.0001	< 0.0001	< 0.0001
PGF2α synthase	< 0.0001	< 0.0001	< 0.0001
TMEM26	< 0.0001	< 0.0001	< 0.0001
Fig 4B			
UCP1	0.0012	< 0.0001	< 0.0001
Elovl3	0.0629	0.019	< 0.0001
Cidea	0.2104	< 0.0001	< 0.0001
CPT1b	0.0002	< 0.0001	< 0.0001
Cox8b	< 0.0001	0.0019	0.0003
PPARα	< 0.0001	< 0.0001	< 0.0001
COX2	< 0.0001	0.0003	< 0.0001
PGF2α synthase	< 0.0001	< 0.0001	< 0.0001
TMEM26	< 0.0001	< 0.0001	0.0175
Fig 6A			
MCP1	< 0.0001	< 0.0001	< 0.0001
TNFα	< 0.0001	< 0.0001	< 0.0001
F4/80	< 0.0001	< 0.0001	< 0.0001
IL6	< 0.0001	< 0.0001	< 0.0001
Arginase 1	< 0.0001	< 0.0001	< 0.0001
Mrc 1	< 0.0001	< 0.0001	< 0.0001
Clec 10	< 0.0001	< 0.0001	< 0.0001
Fig 6B			
MCP1	< 0.0001	< 0.0001	< 0.0001
TNFα	< 0.0001	< 0.0001	0.0001
F4/80	< 0.0001	< 0.0001	< 0.0001
IL6	< 0.0001	0.1794	0.0003
Arginase 1	0.2374	0.2133	0.4565
Mrc 1	< 0.0001	0.0535	< 0.0001
Clec 10	< 0.0001	0.1012	0.0028

Dependent variable	Type	Dose	Interaction
Fig 7A			
PPARγ	< 0.0001	< 0.0001	< 0.0001
Perilipin	< 0.0001	0.0093	< 0.0001
FABP4	< 0.0001	0.2678	< 0.0001
HSL	0.0004	< 0.0001	< 0.0001
ATGL	0.0007	< 0.0001	< 0.0001
Fig 7B			
PPARγ	0.8922	< 0.0001	0.0002
Perilipin	< 0.0001	< 0.0001	< 0.0001
FABP4	0.0039	0.0173	< 0.0001
HSL	0.0398	< 0.0001	0.0012
ATGL	0.7346	< 0.0001	0.001
Fig 8A			
GPR120	< 0.0001	0.313	< 0.0001
GPR40	0.0443	0.3533	0.0132
Fig 8B			
GPR120	0.5264	< 0.0001	< 0.0001
GPR40	< 0.0001	< 0.0001	0.0433
Suppl Fig 1			
UCP1	0.8595	0.0022	0.1126
Elovl3	< 0.0001	< 0.0001	< 0.0001
Cidea	0.0792	< 0.0001	< 0.0001
CPT1b	0.0012	< 0.0001	0.0001
Cox8b	< 0.0001	< 0.0001	< 0.0001
PPARα	0.0033	< 0.0001	< 0.0001
COX2	0.0013	< 0.0001	< 0.0001
PGF2α synthase	< 0.0001	0.0011	0.0422
TMEM26	< 0.001	< 0.001	< 0.001
Suppl Fig 2			
MCP1	< 0.0001	< 0.0001	< 0.0001
TNFa	< 0.0001	< 0.0001	< 0.0001
F4/80	< 0.0001	< 0.0001	< 0.0001
IL6	< 0.0001	< 0.0001	< 0.0001
Arginase 1	< 0.0001	< 0.0001	< 0.0001
Mrc 1	< 0.0001	< 0.0001	< 0.0001
Clec 10	< 0.0001	0.036	0.0164
Suppl Fig 3			
PPARg	0.0466	< 0.0001	< 0.0001
Perilipin	< 0.0001	< 0.0001	< 0.0001
FABP4	0.0003	< 0.0001	< 0.0001
HSL	0.0631	< 0.0001	0.0001
ATGL	0.0368	< 0.0001	0.0022
Suppl Fig 4			
GPR120	< 0.0001	< 0.0001	< 0.0001
GPR40	0.0044	0.0229	0.0635

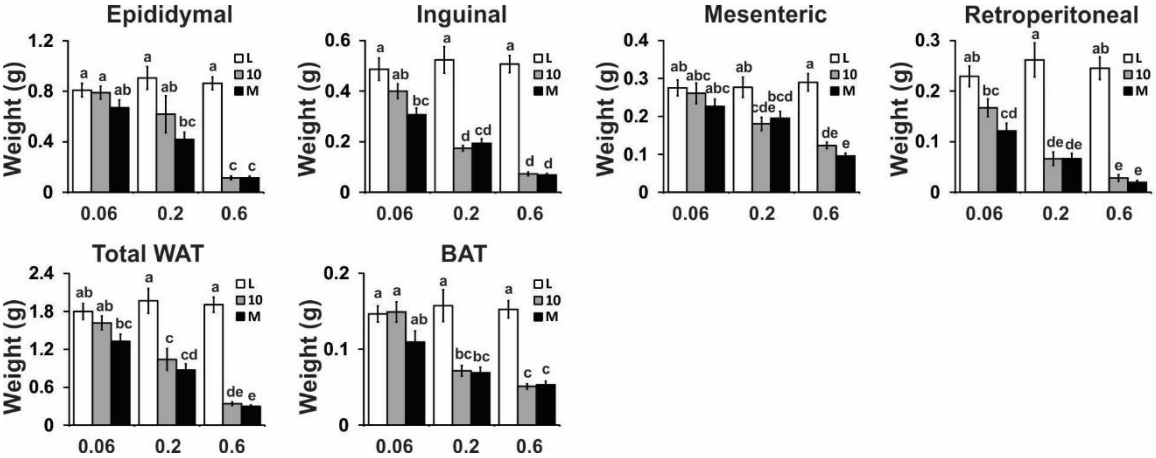


Figure 4.1 Intermediate and High Doses of CLA Treatments Decrease Adiposity. Young, male 129SV mice (N = 10 per treatment group) were fed for 7 weeks a standard, purified mouse diet containing 0.06, 0.2, or 0.6% linoleic acid control (L), 10,12 CLA plus linoleic acid (10), or 10,12 CLA plus 9,11 CLA (M). Epididymal, inguinal, retroperitoneal, mesenteric, and BAT depots were excised and weighed. Means ± SEM without a common letter differ ($P < 0.05$).

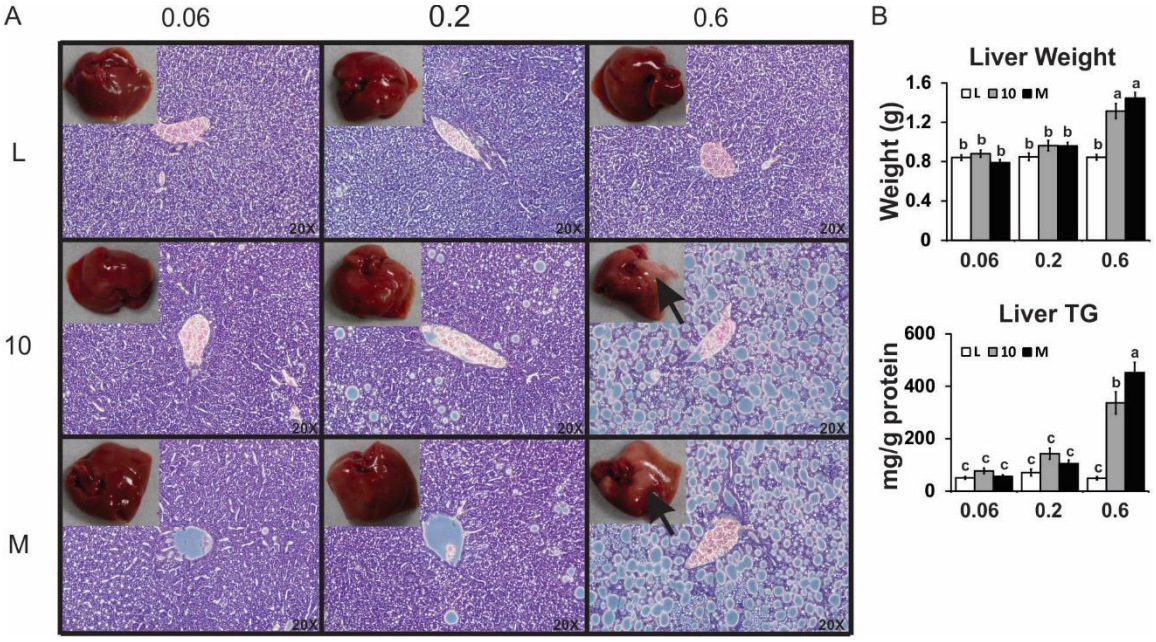


Figure 4.2 High Dose of CLA Treatments Cause Hepatic Steatosis and Hepatomegaly. A: Livers were excised and weighed, and then a section of liver was fixed in formalin, sliced, and stained with hematoxylin and eosin. B: Liver weights were recorded, liver TG and protein contents were measured, and the ratio of TG per milligram of protein was used as an indicator of steatosis. Means ± SEM without a common letter differ ($P < 0.05$). L, linoleic acid control; 10, 10,12 CLA plus linoleic acid; M, 10,12 CLA plus 9,11 CLA.

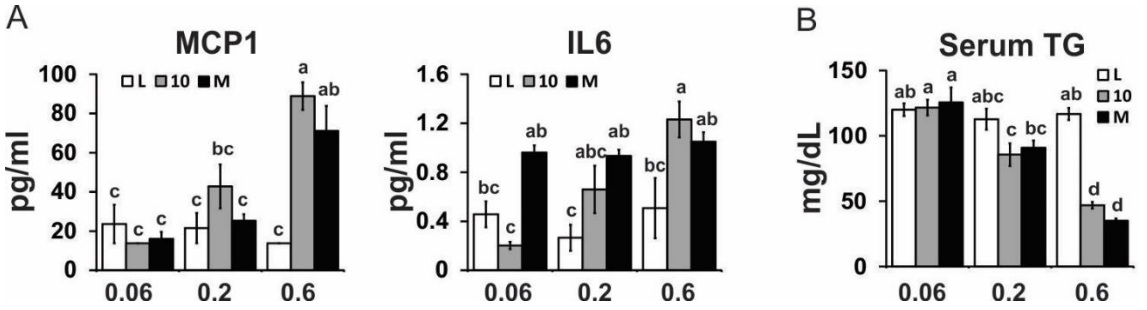


Figure 4.3 High Dose of CLA Treatments Increases Serum MCP-1 Levels. A: Serum levels of MCP-1 and IL-6. B: Serum TG levels. Means ± SEM without a common letter differ ($P < 0.05$). L, linoleic acid control; 10, 10,12 CLA plus linoleic acid; M, 10,12 CLA plus 9,11 CLA.

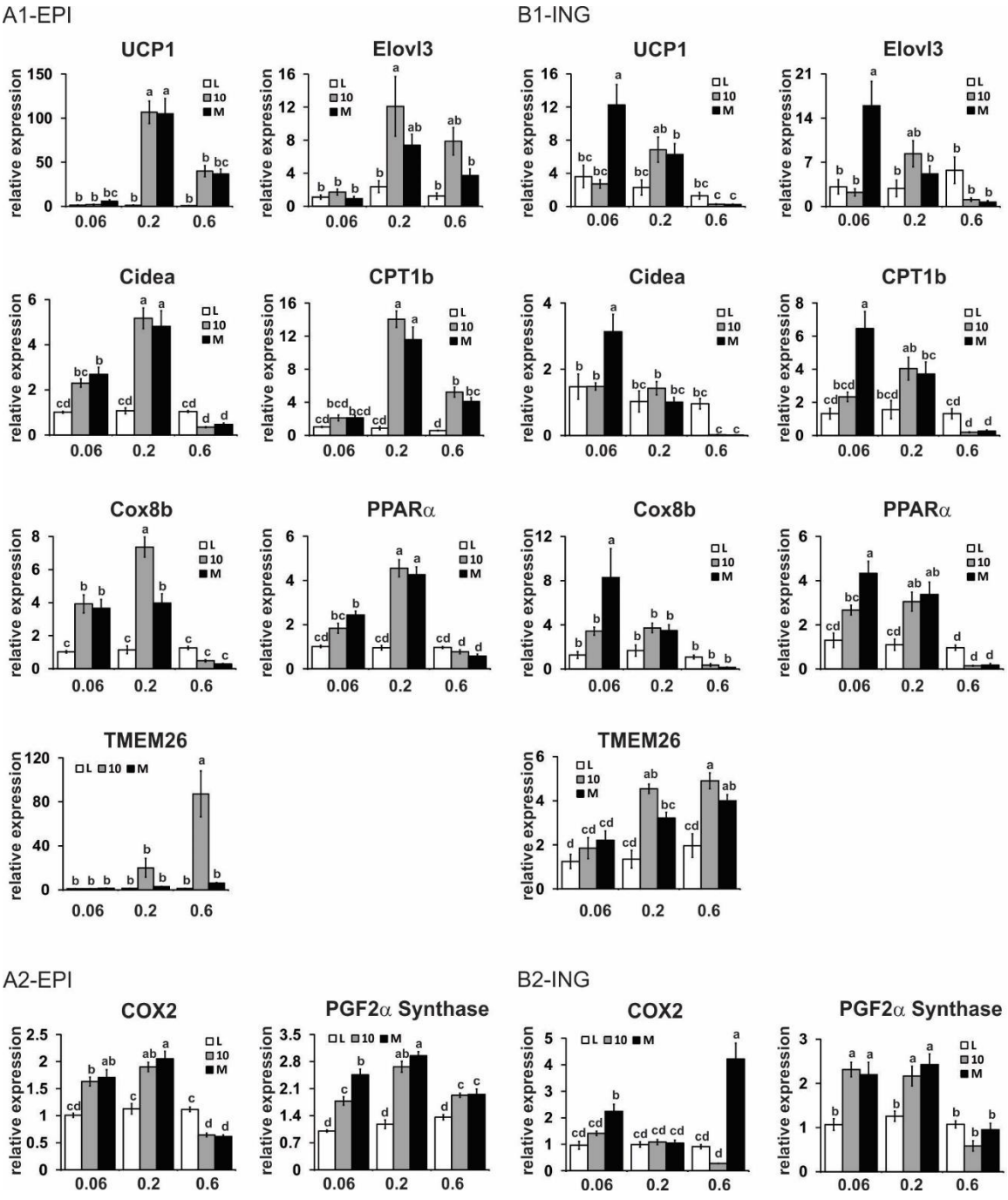


Figure 4.4 Intermediate Dose of CLA Treatments Increases Markers of Browning in Epididymal (EPI) WAT. mRNA levels of markers of brown fat-like adipocytes were measured in EPI (A) or inguinal (ING) (B) WAT by real-time qPCR. (A1) and (B1) are direct markers of browning, and (A2) and (B2) are markers of prostaglandin production that are

associated with the activation of browning. Means ± SEM without a common letter differ ($P < 0.05$). L, linoleic acid control; 10, 10,12 CLA plus linoleic acid; M, 10,12 CLA plus 9,11 CLA.

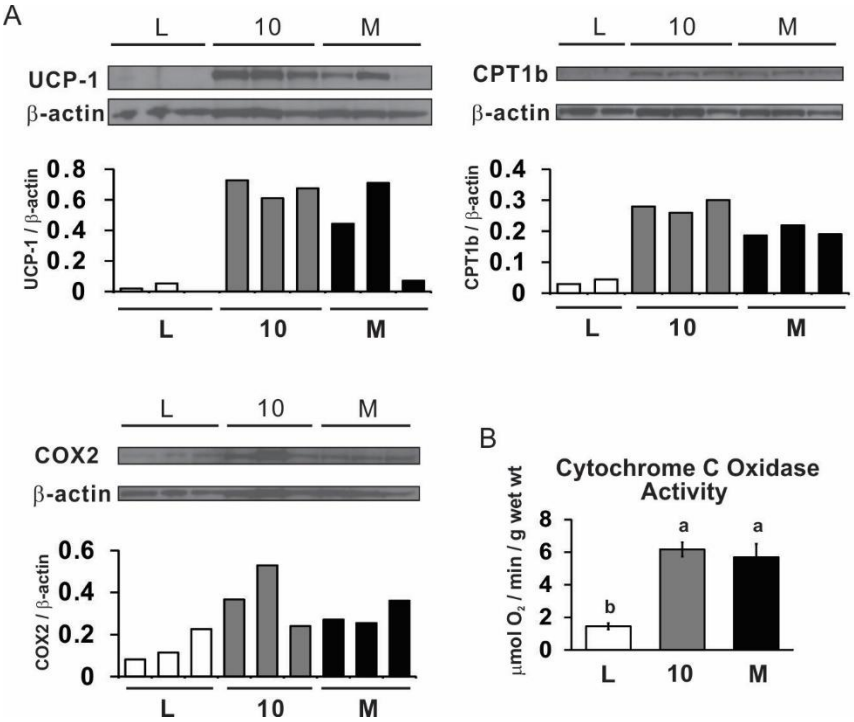


Figure 4.5 Intermediate Dose (0.2%) of CLA Treatments Increases the Protein or Activity Levels of Browning Markers in Epididymal (EPI) WAT. A: Protein levels of UCP1, CPT-1b, COX-2, and β-actin (load control) were measured in homogenates of EPI WAT. B: Activity of cytochrome c oxidase was measured in homogenates of EPI WAT. Means ± SEM without a common letter differ ($P < 0.05$). L, linoleic acid control; 10, 10,12 CLA plus linoleic acid; M, 10,12 CLA plus 9,11 CLA.

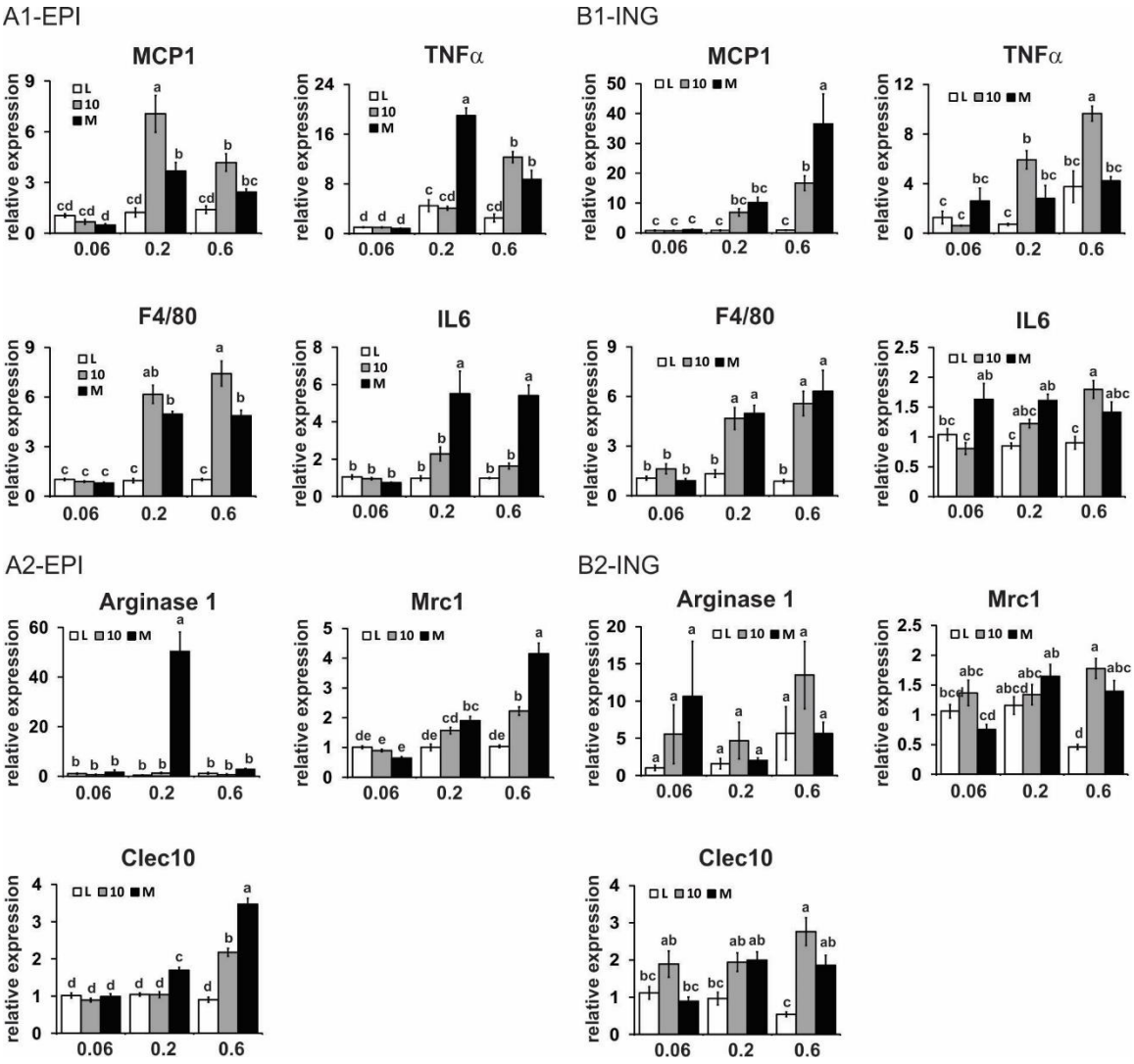


Figure 4.6 CLA Treatments Increase mRNA Markers of Inflammation in Epididymal (EPI) and Inguinal (ING) WAT. mRNA levels of markers of low-grade inflammation were measured in EPI (A) and ING (B) WAT by real-time qPCR. (A1) and (B1) are markers of classically activated M1 macrophage markers, and (A2) and (B2) are alternatively activated M2 macrophage markers. Means ± SEM without a common letter differ ($P < 0.05$). L, linoleic acid control; 10, 10,12 CLA plus linoleic acid; M, 10,12 CLA plus 9,11 CLA; Arg1, arginase-1; Mrc1, mannose receptor c1.

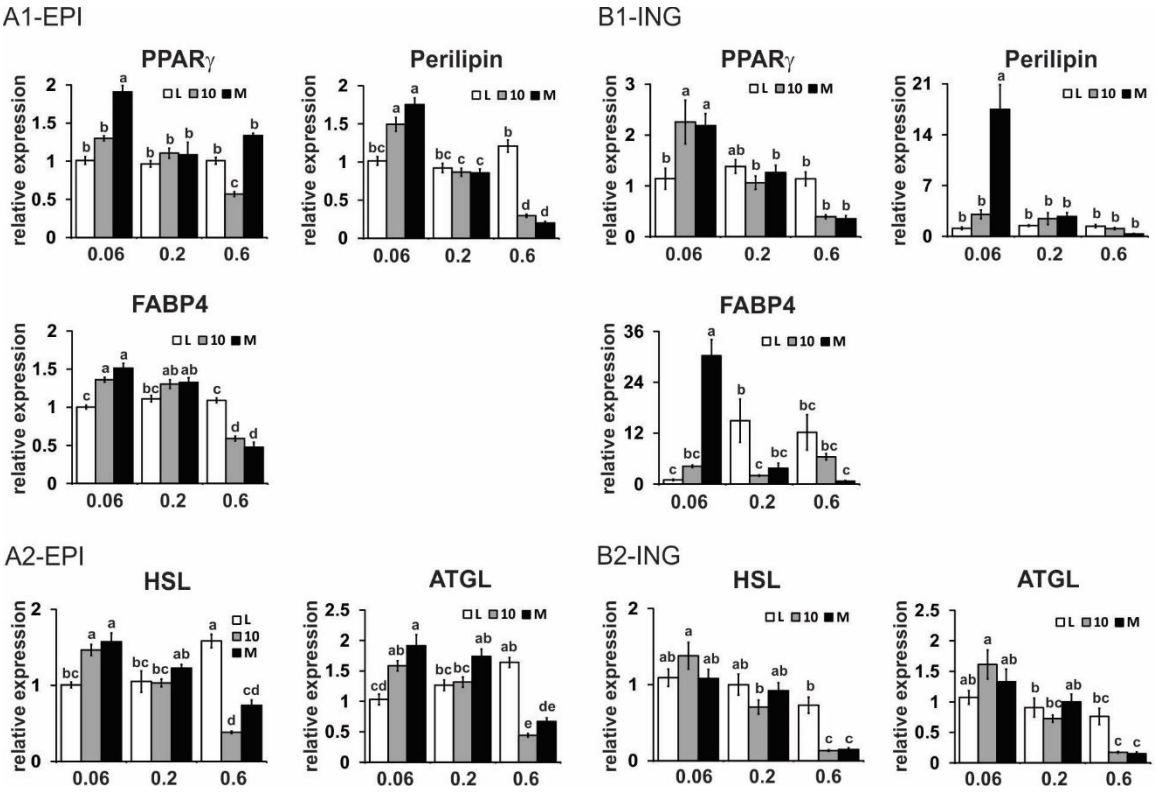


Figure 4.7 Impact of CLA Treatment on mRNA Markers of Lipogenesis and Lipolysis in Epididymal (EPI) and Inguinal (ING) WAT. mRNA levels of markers of lipogenesis (A1, B1) and lipolysis (A2, B2) were measured in EPI (A) and ING (B) WAT by real-time qPCR. Means ± SEM without a common letter differ ($P < 0.05$). L, linoleic acid control; 10, 10,12 CLA plus linoleic acid; M, 10,12 CLA plus 9,11 CLA; HSL, hormone sensitive lipase; ATGL, adipose tissue TG lipase.

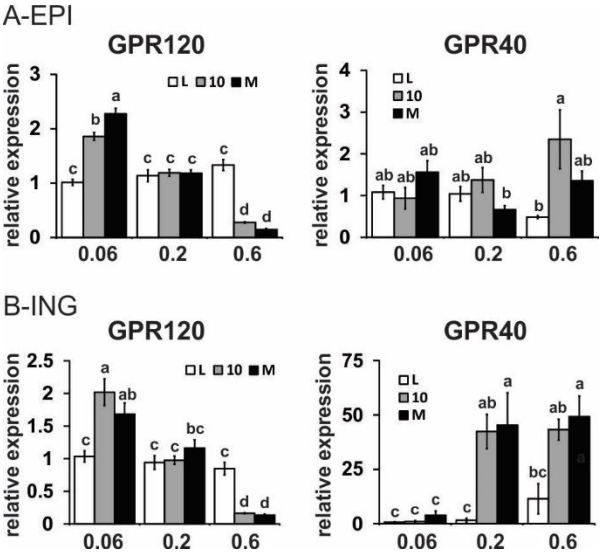
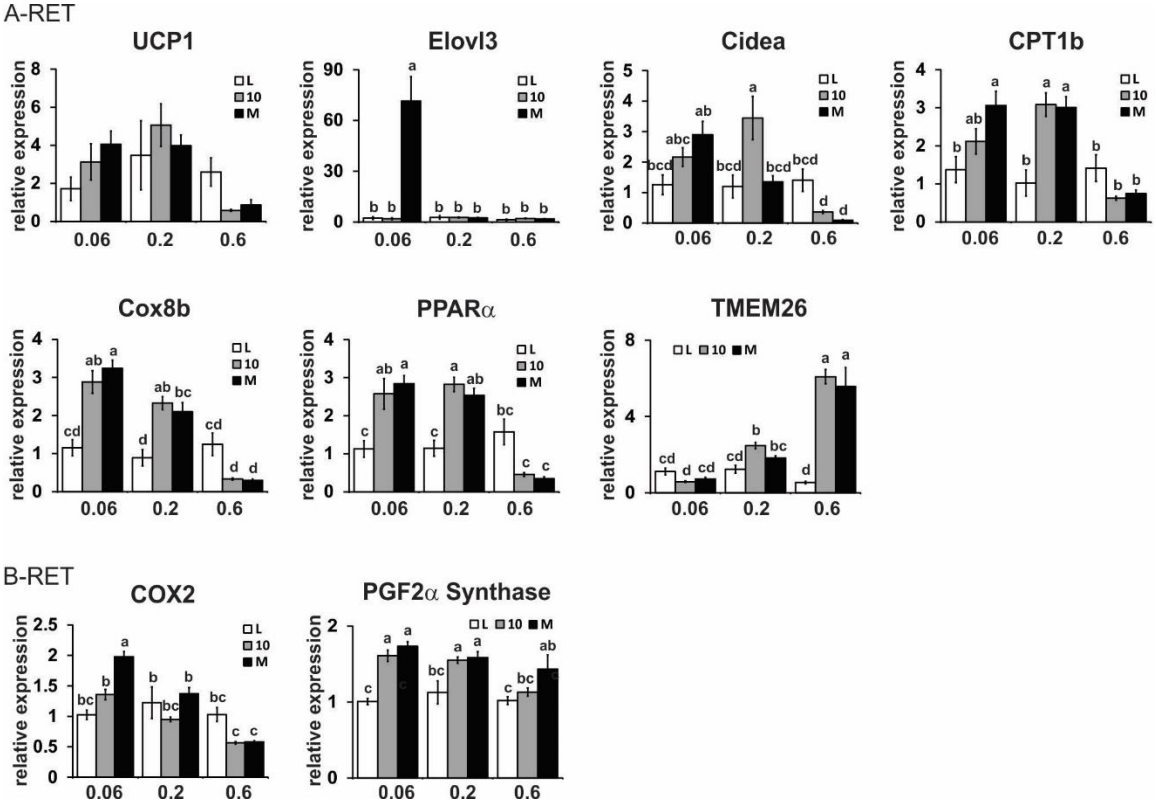
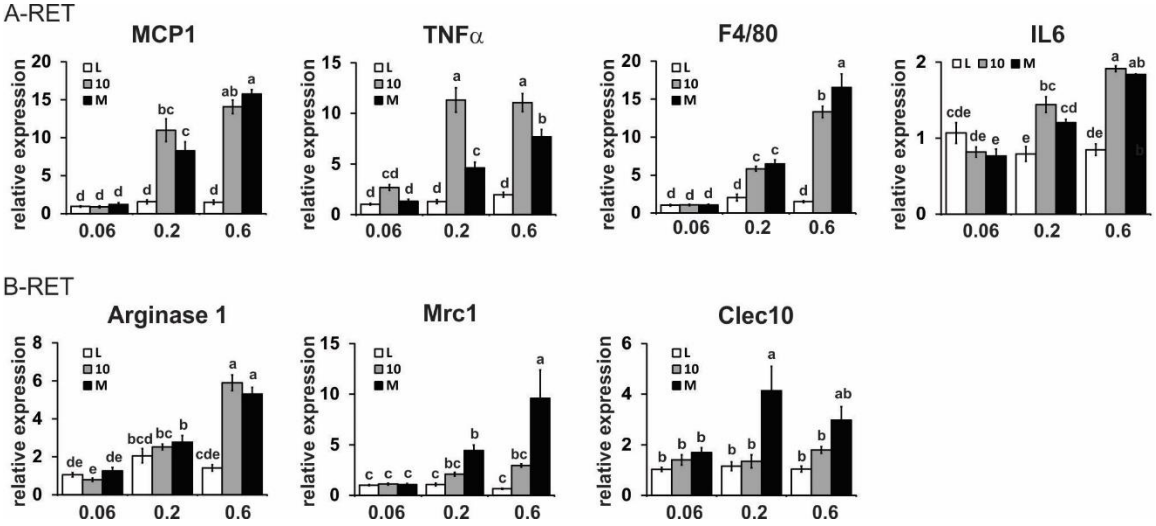


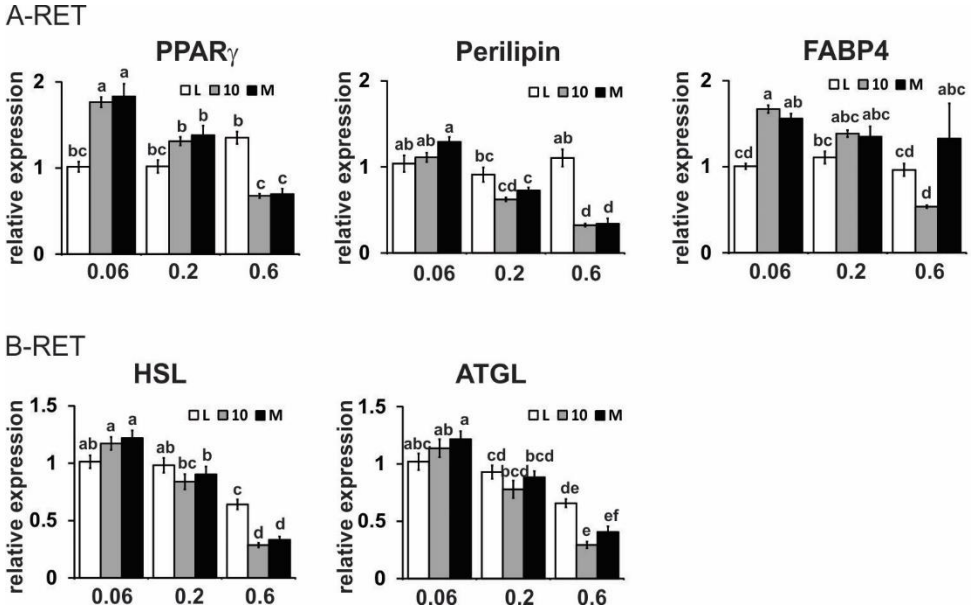
Figure 4.8 FFA Receptors GPR120 and GPR40 in Epididymal (EPI) and Inguinal (ING) WAT are Differentially Regulated by CLA. mRNA levels of markers of GPR 120 and GPR40 in EPI (A) and ING (B) were measured by real-time qPCR. Means ± SEM without a common letter differ ($P < 0.05$). L, linoleic acid control; 10, 10,12 CLA plus linoleic acid; M, 10,12 CLA plus 9,11 CLA.



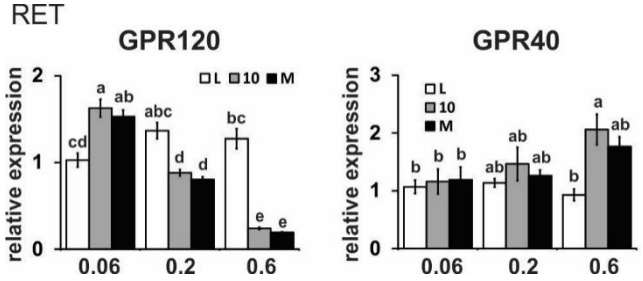
Supplemental Figure 4.1 Differential Effects of CLA Dose and Treatments on Markers of Browning in Retroperitoneal (RET) WAT. Panel A; direct markers of browning. Panel B; markers of prostaglandin production that are associated with the activation of browning. mRNA levels of markers of brown fat-like adipocytes were measured in retroperitonial WAT by real time qPCR. Means + SEM without a common letter differ ($P<0.05$). L, linoleic acid; 10, 10,12 CLA plus linoleic acid; M, 10,12 CLA plus 9,11 CLA; UCP-1, uncoupling protein 1; Elovl3, elongation of very long chain fatty acids 3; Cidea, cell death-induced DNA fragmentation factor-a-like effector A; CPT-1b, carnitine palmitoyltransferase 1 b; Cox8b, cytochrome c oxidase subunit VIII b; PPARα, proliferator-activated receptor alpha; TMEM26, transmembrane protein 26; COX-2, cyclooxygenase-2; PGF2α,prostaglandin F2 alpha.



Supplemental Figure 4.2 Intermediate and High Dose of CLA Treatments Increase Marker of Low Grade Inflammation in Retroperitoneal WAT. Panel A; markers of classically-activated M1 macrophage. Panel B; markers of alternatively-activated M2 macrophage. mRNA levels of markers of low grade inflammation were measured in retroperitonial WAT by real time qPCR. Means + SEM without a common letter differ ($P<0.05$). L, linoleic acid; 10, 10,12 CLA plus linoleic acid; M, 10,12 CLA plus 9,11 CLA; COX-2, cyclooxygenase 2; MCP-1, monocyte chemoattractant protein 1; IL-6 , interleukin 6; TNFα = tumor necrosis factor alpha.



Supplemental Figure 4.3 Differential Effects of CLA Dose and Treatments on Markers of Lipogenesis and Lipolysis in Retroperitoneal WAT. mRNA levels of markers of lipogenesis (Panel A) and lipolysis (Panel B) were measured in retroperitoneal WAT by real time qPCR. Means + SEM without a common letter differ ($P<0.05$). L, linoleic acid; 10, 10,12 CLA plus linoleic acid; M, 10,12 CLA plus 9,11 CLA; PPARα, peroxisome proliferator-activated receptor alpha; FABP4, fatty acid binding protein 4,; HSL, hormone sensitive lipase; ATGL, adipose tissue TG lipase.



Supplemental Figure 4.4 FFA Receptors GPR120 and 40 in WAT are Differentially Regulated by CLA. mRNA levels of markers of GPR 120 and 40 were in RET WAT measured by real time qPCR. Means + SEM without a common letter differ ($P<0.05$). L, linoleic acid; 10, 10,12 CLA plus linoleic acid; M, 10,12 CLA plus 9,11 CLA; GPR, G protein receptor.

CHAPTER V

LOW LEVEL OF TRANS-10, CIS-12 CONJUGATED LINOLEIC ACID DECREASES ADIPOSITY AND INCREASES BROWNING INDEPENDENT OF INFLAMMATORY SIGNALING IN OVERWEIGHT SV129 MICE

Formatted for Shen W, Baldwin J, Collins B, Hixson L, Lee KT, Herberg T, et al. (2015). *Journal of Nutrition Biochemystry, 26(6),* 616-25.

Abstract

The objective of this study was to determine the extent to which a low level of trans-10, cis-12 (10,12) conjugated linoleic acid (CLA) decreases adiposity and increases browning in overweight mice, its dependence on inflammatory signaling and potential synergistic effects of daily exercise. Young, Sv129 male mice were fed a high-fat diet for 5 weeks to make them fat and glucose intolerant and then switch them to a low-fat diet with or without 0.1% 10,12 CLA, sodium salicylate or exercise for another 7 weeks. 10,12 CLA decreased white adipose tissue (WAT) and brown adipose tissue mass, and increased the messenger RNA and protein levels, and activities of enzymes associated with thermogenesis or fatty acid oxidation in WAT. Mice fed 10,12 CLA had lower body temperatures compared to controls during cold exposure, which coincided with decreased adiposity. Although sodium salicylate decreased 10,12 CLA-mediated increases in markers of inflammation in WAT, it did not affect other outcomes. Exercise had no further effect on the outcomes measured. Collectively, these data indicate that 10,12 CLA-mediated reduction of adiposity is independent of inflammatory signaling, and possibly due to up-regulation of fatty acid oxidation and heat production in order to

regulate body temperature. Although this low level of 10,12 CLA reduced adiposity in overweight mice, hepatomegaly and inflammation are major health concerns.

Introduction

The prevalence of overweight and obesity has reached epidemic proportions over the past two decades. In 2010, 35.7% of American adults and 17% of children or adolescents were obese (1). In 2012, 13 states in the United States had obesity rates greater than 30% (2). The direct annual economic loss due to disability, injury and death from being overweight and obese in the United States was ~170 billion dollars (3). Indirect medical costs from obesity-related chronic diseases including cardiovascular disease, renal disease and diabetes further increased heath care costs (3). Therefore, developing long-term, effective strategies to decrease the prevalence of obesity and its comorbidities are urgently needed.

Conjugated linoleic acid (CLA) supplementation has become a popular method for weight management, especially after it was approved by the Food and Drug Administration for Generally Recognized as Safe status in 2008. Proposed mechanisms by which CLA, particularly the trans-10, cis-12 (10,12) isomer, reduces adiposity include regulation of energy metabolism, adipogenesis, lipid metabolism, white adipose tissue (WAT) apoptosis and inflammation (reviewed in Ref. (4)). Studies have linked 10,12 CLA-mediated inflammatory signaling to the suppression of adipogenesis (5,6), lipogenesis (5–7), insulin sensitivity (5,6,8), and induction of lipolysis (5) and apoptosis (9).

We previously demonstrated that 10,12 CLA increased the expression of several G-coupled receptor proteins (GPR) such as GPR56 and GPRC5A (10), and activated

phospholipase c (11) and diacyglycerol kinase (12), resulting in increased calcium release from endoplasmic reticulum (ER) (13) in human primary adipocytes. By chemically blocking calcium release from the ER, 10,12 CLA-mediated activation of extracellular signal-regulated kinase (ERK)1/2 (6,14), cJun-NH2-terminal kinase (15) and nuclear factor kappa B (NF-κB) (8) were attenuated (13). Notably, these 10,12 CLA-activated proteins antagonized peroxisome proliferator-activated receptor (PPAR) γ and its target genes (5,6,8,14,15), which contributed to CLA's reduction of adipocyte triglyceride (TG) content. 10,12 CLA-mediated increase in intracellular calcium also impaired insulin sensitivity (13) and impacted prostaglandin synthesis (13,16).

Notably, increased thermogenesis driven by inflammatory signaling has recently been reported to prevent diet-induced obesity (17–20). For example, cyclooxygenase (COX)-2 transgenic mice had an increased abundance of mitochondria in WAT, expression of uncoupling protein (UCP) 1 in WAT and systemic energy expenditure (17). By knocking out COX-2, UCP1 expression in WAT was suppressed and the defense of body temperature during chronic cold exposure was impaired in mice (17). Notably, overexpressing NF-κB was shown to prevent high fat-induced obesity by elevated energy expenditure through thermogenesis (19,20).

Our previous study demonstrated that a low dose of 10,12 CLA (0.1%) prevented an accumulation of body fat in young Sv129 mice fed a low-fat diet for 7 weeks (21). These CLA-mediated changes in body fat were associated with increased messenger RNA (mRNA) and protein levels of markers associated with browning and inflammation in epididymal WAT (21). Therefore, the objective of this study was to determine the extent to which this low dose of 10,12 CLA reduced adiposity in mice made overweight through feeding an American-type, high-fat diet and then transitioned to a low-fat diet,

and its dependence on inflammatory signaling. In addition, we wanted to determine if daily aerobic exercise could (i) enhance 10,12 CLA's decrease in adiposity and (ii) decrease 10,12 CLA's potential increase in hepatic TG levels.

Methods

Experimental design and diets

Ninety, 4- to 6-week-old, Sv129 male mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and housed in pairs in a 12-h light/12-h dark, temperature-controlled (22°C) animal facility at University of North Carolina at Greensboro. Ethical treatment of animals was assured by the campus Institutional Animal Care and Use Committee. Sv129 mice were used based on their capacity to develop beige adipocytes compared to C57BL6 mice (18). After 1 week of acclimation to the animal facility, 80 mice were fed a high-fat diet (i.e., 35% kcal from fat; see Supplemental Table 5.1 and Figure 5.1) for 5 weeks (phase 1=weeks 1–5, fattening period). After 5 weeks of high-fat feeding, 10 mice were maintained on this high-fat diet (HH) for the remaining 7 weeks of the study. The other 70 mice were randomly assigned to either low fat (i.e., 10% kcal from fat, HL; Supplemental Table 5.1) with (HL+C; n=12) or without 0.1% 10,12 CLA (HL; n=10), low fat combined with exercise (E) with (HLE+C; n=12) or without 10,12 CLA (HLE; n=12), or low fat combined with 4 g/kg sodium salicylate (S) with (HLS+C; n=12) or without 10,12 CLA (HLS; n=12) for another 7 weeks. The remaining 10 mice were fed a low-fat diet throughout the 12-week study (LL; 10% kcal from fat; Supplemental Table 5.1). The experimental design is illustrated in Figure 5.1. The length of this study and the dose and isomer of CLA were chosen based on the results of our previous study (21). 10,12 CLA (purity=98%) was purchased from

Matreya LLC (Pleasant Gap, PA, USA). Sodium salicylate, an anti-inflammatory agent that has been shown to suppress NF-κB signaling in vitro (22) and in vivo (23,24), was purchased from Sigma-Aldrich (St. Louis, MO, USA). The dose of sodium salicylate was based on work by Yuan et al. (23) and Herrero et al. (24). Diets were prepared by Research Diets (New Brunswick, NJ, USA) and stored at −20°C until use. Mice had ad libitum access to both food and water, and food was changed every 3–4 days. Food intake and body weight were measured weekly.

At the end of the study, fasted mice were euthanized with isoflurane vapor followed by cervical dislocation. Blood (serum), WAT depots (i.e., epididymal, inguinal, mesenteric and retroperitoneal), brown adipose tissue (BAT), gastrocnemius muscle and liver were harvested, weighted and frozen in liquid N_2 and stored at −80°C until analyses.

Intraperitoneal glucose tolerance tests

Intraperitoneal glucose tolerance tests (GTTs) were conducted on nonanesthetized mice at weeks 5 and 11. Mice were deprived of food for 8 h and given an intraperitoneal glucose injection (i.e., 20% glucose solution) at a dose of 1 g/kg body weight on the day of test. One drop of venous blood was taken from a small tail clip, and blood glucose was measured using a Contour blood glucometer (Bayer Diabetes Care, Tarrytown, NY, USA) at 0, 5, 15, 30, 60 and 120 min after glucose administration. Total area under the curve (AUC) for the GTT was calculated as described (25).

Dual-energy x-ray

In order to measure percentage body fat, dual-energy x-ray (DEXA) measurements were performed using a GE Lunar Prodigy Advanced System (GE Healthcare, Milwaukee, WI, USA) at weeks 5 and 11. Mice were lightly anesthetized with isoflurane vapor using a SomnoSuite Small Animal Anesthesia System (Kent Scientific, Torrington, CT, USA) with Integrated Digital Vaporizer isoflurane system and then positioned on the DEXA table with appendages extended away from the body. The system was calibrated daily according to the manufacturer's instructions prior to scans. DEXA measurements were done in duplicate for each mouse. Data were analyzed using Encore 2007 Small Animal software (version 11.20.068).

Body temperatures

During the first week of acclimatization to the animal facility, mice were implanted subcutaneously with microtransponders (BHMDS IPTT-300) purchased from Bio Medic Data Systems (BMDS, Seaford, DE, USA) while under isoflurane vapor anesthesia. The sites of insertion were examined daily for 5 days post-implant to ensure that there were no signs of infection. At weeks 8 and 11, mice from all treatments were exposed to cold temperature (7°C) for 4 h, and body temperatures were measured at baseline and every hour for 4 h (26).

Exercise protocol

Exercise started on week 6 on a 5-day/week basis at room temperature. Mice were trained on a motor-driven rodent treadmill (Collins Instruments, Braintree, MA, USA) equipped with a Coulbourne Precision Regulated Animal Shocker at the back.

Very low current was used to encourage animals to run. Mice in the exercise groups were familiarized with the treadmill each day for 1 week by gradually increasing running time and speed, so that at the end of 1 week, they were running for 30 min at a 10-m/min pace for the first 15 min and 12 m/min for the second 15 min up a 12% grade. Following habituation, the length of each exercise session was gradually increased 5 min/day until the animals were running for 1 h per day at a 12-m/min pace up a 12% grade. No fatigue was observed during the study as evidenced by not getting shocked more than four times in a minute or staying by the shocker for more than 5 s (27). This duration and intensity were maintained for the remainder of the exercise protocol. This moderate protocol has been reported to prevent high fat-mediated steatosis (28) and increase mitochondria content in leg muscles of C57BL/6 mice (29).

Cytochrome c oxidase and 3-hydroxyCoA dehydrogenase activities

Cytochrome c oxidase, the final protein complex in the electron transport chain, and 3-hydroxyCoA dehydrogenase (3-HAD), a marker enzyme of β-oxidation, were used as indicators of mitochondria content. Epididymal and inguinal fat samples were homogenized and the activities of cytochrome c oxidase (30) and 3-HAD (31) were measured as previously described (21).

Liver TG content

Liver TG levels were measured as previously described (21). Liver protein concentration was determined by using Lowry protein assay from delipidated liver.

Tissue RNA extraction and real-time quantitative polymerase chain reaction

Total mRNA was extracted from WAT tissue using the RNeasy Lipid Tissue Kit (Qiagen, Valencia, CA, USA), followed by using the DNase treatment (Qiagen). Single-strand RNA was reverse-transcribed into complementary DNA (cDNA) using the high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA), and real-time quantitative polymerase chain reaction (qPCR) was performed in 7500 FAST Real Time PCR System as previously described (21). TATA-binding protein (TBP) was used as the internal control.

Apoptosis PCR Array

RT2 Profiler PCR Array kit (Qiagen) for apoptosis was conducted on epididymal mRNA samples. Procedures were followed using the company's instructions. Briefly, total RNA samples were first tested for degradation on a 1% agarose gel, and then 1 μg of mRNA was reverse transcribed to cDNA using the Qiagen's TR^{2}First Strand Kit. Equal aliquots of cDNA were added to a PCR array plate which was coded with 84 genes at the bottom of each well. Real-time amplification data were collected using the 7500 FAST Real Time PCR System. Data analysis was conducted using Web-based data analysis software provided by the company.

Immunoblotting

Immunoblotting was conducted as previously described (21) using primary antibodies of UCP-1 (#ab23841; Abcam, Cambridge, MA, USA), carnitine palmitoyltransferase 1b (#sc20670; CPT1b; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and β-actin (#sc1616; Santa Cruz) at 1:400, 1:200 and 1:1000 dilution,

respectively. Horseradish peroxidase-conjugated secondary antibodies were probed at 1:1000 dilutions at room temperature for 2 h. Blots were exposed to chemiluminescence reagent, and x-ray films were developed using a SRX-101A Konica Minolta film developer. Two samples from HL, HL+CLA, HLS and HLS+CLA treatments were randomly selected and run on the same gel and are representative of other samples within each treatment group. Densitometry was conducted using a Kodak 4400 CF Image Station as described previously (21).

Statistics

Student's t test was performed to compare (i) the effects of high-fat vs. low-fat feeding during the first 5-week period of the study and (ii) the effects of HL vs. HL+CLA on the body temperature and body fat percentage data at weeks 8 and 11 (Pb.05). A one-way analysis of variance (ANOVA), Tukey's HSD multicomparison was conducted to detect significant treatment differences in (i) weights of adipose tissues, (ii) mRNA, protein and enzyme activity levels of browning markers, (iii) mRNA levels of inflammatory markers and (iv) liver weight and TG content. We also used Bonferroni's post hoc test to perform specific comparisons (i.e., HL vs. HL+CLA, HLE vs. HLE+CLA, HLS vs. HLS+CLA, HL+CLA vs. HLE+CLA and HL+CLA vs. HLS+CLA groups), five groups in all using a family error rate of 0.05. Hence, differences were considered significant at Pb.01 for Bonferroni's adjustment. All statistical analyses were conducted by the JMP version 8.0 program (SAS, Cary, NC, USA). Data are expressed as means ± standard error of the mean (S.E.M.).

Results

High-fat diet increases body weight, body fat and insulin resistance at week 5 (phase 1, fattening period; weeks 1–5)

After 1 week on the experimental diets, mice fed the high-fat diet had higher body weights compared to mice fed the low-fat diet (Figure 5.2A). At weeks 5, high-fat-fed mice had a greater percentage of body fat (Figure 5.2B) and increased fasting blood glucose level (Figure 5.2C) and GTT AUC (Figure 5.2D) compared to the low fat-fed mice. Collectively, these data demonstrated that the fattening period (phase 1) was of sufficient length to increase adipose tissue mass and cause glucose tolerance.

10,12 CLA decreases adiposity without impacting glucose tolerance at weeks 11–12 (phase 2, CLA treatment period; weeks 6–12)

Mice fed the high-fat diet for 12 weeks had the highest body weights (Supplemental Table 5.2), and visceral (i.e., epididymal and mesenteric) and subcutaneous (i.e., inguinal) WAT depot weights (Figure 5.3) compared to mice fed the low fat-fed mice (LL) for 12 weeks and mice fed the high-fat diet for 5 weeks and then switched to the low-fat diet for 7 weeks (HL control group). All 10,12 CLA-treated mice had decreased weights of visceral (i.e., epididymal and retroperitoneal) and subcutaneous (i.e., inguinal) WAT and BAT compared to the mice fed the high-fat diet for 5 weeks and then reduction in adiposity. In addition, none of the treatments (i.e., switched to the low-fat diet for 7 weeks (HL controls; Figure 5.3). Neither 10,12 CLA, exercise or sodium salicylate) impacted glucose tolerance exercise nor sodium salicylate treatment affected 10,12 CLA's levels compared to their controls (Supplemental Table 5.2).

10,12 CLA decreases body temperature during cold exposure (phase2, CLA treatment period; weeks 6–12)

At weeks 8 and 11, 10,12 CLA-treated mice had an impaired ability to maintain body temperature after exposure to 7°C for 4 h compared to the mice fed the high-fat diet for 5 weeks and then switched to the low-fat diet for 7 weeks (HL control group; Figure 5.4A and 5.4B). This reduction in body temperature was not observed in 10,12 CLA-treated mice that were exercised or supplemented with sodium salicylate (data not shown). Notably, the reduction in body temperature by10,12 CLA at weeks 11 coincided with the reduction in percentage of body fat during the same week (Figure 5.4C), indicating that the loss of body fat may have impacted the mice's ability to defend body temperature during cold exposure.

10,12 CLA increases markers of browning and thermogenesis (phase 2, CLA treatment period; weeks 6–12)

Mice consuming the low-fat and high-fat diets for 12 weeks (LL and HH, respectively) had similar mRNA levels of markers associated with browning in epididymal and inguinal fat depots (Figure 5.5A and 5.5B). Consistent with our previous study (21), all 10,12 CLA-treated mice had increased mRNA levels of markers of thermogenesis and fatty acid oxidation in epididymal (Figure 5.5A) and inguinal (Figure 5.5B) WAT including UCP1 (uncouples phosphorylation of ADP to ATP), CPT1b (facilitates fatty acid transport into mitochondria), cytochrome c oxidase VIII b (Cox8b; promotes electron transfer during mitochondrial respiration) and PPARα (induces genes associated with fatty acid oxidation) compared to the HL controls. This 10,12 CLA-mediated induction was greatest in the epididymal depot, consistent with our previous

study (21). Exercise did not further affect markers of browning in control or 10,12 CLA-treated mice. In contrast to our hypothesis, sodium salicylate did not prevent 10,12 CLA's induction of mRNA markers of browning in either WAT depot.

Consistently, protein levels of UCP1 and CPT1b were higher in 10,12 CLA-treated mice in visceral (epididymal) and subcutaneous (inguinal) depots compared to their respective controls, and sodium salicylate did not attenuate this increase by 10,12 CLA (Figure 5.6). In agreement with mRNA and protein data, the activities of cytochrome coxidase and 3-HAD (Figure 5.7A) were elevated by 10,12 CLA treatment compared to the controls in the epididymal depot. Exercise did not further elevate the activity of these two enzymes, nor did sodium salicylate attenuate their activities. Notably, none of the treatments influenced the activities of cytochrome c oxidase or 3-HAD in gastronemius muscle (i.e., indicators of nonshivering thermogenesis; data not shown) or the mRNA levels of UCP1 in BAT (data not shown).

10,12 CLA increases markers of inflammation (phase 2, CLA treatment period; weeks 6–12)

Mice consuming the low-fat and high-fat diets for 12 weeks (LL and HH, respectively) had similar mRNA levels of markers associated with inflammation in epididymal (Figure 5.8A) and inguinal (Figure 5.8B) WAT depots. 10,12 CLA-treated (HL+CLA) mice had increased mRNA levels of markers of inflammation in epididymal and inguinal WAT depots, including those associated with prostaglandin synthesis (e.g., COX-2 and aldose reductase), M1 macrophage responses (e.g., monocyte chemoattractant protein 1 (MCP1), F4/80 and CD11c), and proinflammatory cytokines (e.g., interleukin (IL) 6), compared to the low-fat control mice (HL). Exercise did not

further affect markers of inflammation in either depot. Sodium salicylate attenuated 10,12 CLA-mediated induction of markers of COX-2, MCP1, CD11C, F4/80, tumor necrosis factor (TNF) α and IL6 in the epididymal depot (Figure 5.8).

Mice fed the high-fat diet for 12 weeks (HH) had greater liver weights (Figure 5.9A and 5.9B) and TG content (Figure 5.9C) compared to the mice fed the low-fat diet for 12 weeks (LL). Mice fed 10,12 CLA (HL+CLA) had heavier livers compared to their controls (HL). Surprisingly, exercise (HLE+CLA) or sodium salicylate (HLS+CLA) did not prevent hepatomegaly (Figure 5.9B) or the increase in hepatic TG content (Figure 5.9C) in 10,12 CLA-treated mice. These data indicate that even this low dose (0.1%, wt/wt) of 10,12 CLA may have deleterious effects in the liver, which has been consistently reported by others at higher doses of 10,12 CLA or mixed CLA isomers (32).

Discussion

10,12 CLA-mediated loss of body fat and induction of browning is independent of COX-2 and inflammatory signaling

It has been consistently reported that COX-2 plays an important role in response to classic β3-adrenergic activation from cold exposure or agonists to promote mitochondrial biogenesis and increased UCP1 expression in WAT (17,18,33) (reviewed in Refs. (34,35)). COX-2, the rate-determining enzyme in prostaglandin synthesis, has been shown to be up-regulated in WAT during exposure to cold or stimulation with a β3-adrenergic agonist (17). For example, CL316243-mediated up-regulation of UCP1, cell death-induced DNA fragmentation factor-a-like effector A (Cidea) and CPT1b mRNA levels in intra-abdominal WAT was attenuated by treatment with the selective COX-2 inhibitor celecoxib (17). Our previous study (21) demonstrated that a 7-weeks, low dose

of 10,12 CLA prevented body fat accumulation and increased markers of browning in lean mice, which was accompanied by increased COX-2 mRNA and protein expression.

However, we did not know if this low dose of 10,12 CLA decreased adiposity in overweight mice to a greater extent than consuming a low-fat diet alone, and if so, was it dependent on COX-2 signaling. To answer this question, mice were fed a high-fat diet for 5 weeks to get them overweight and then switched to a low-fat diet with or without CLA and sodium salicylate, a COX-2 inhibitor. Consistent with our hypothesis, all mice receiving 10,12 CLA had lower total WAT depot weights compared to their low fat-fed controls (Figure 5.3). As anticipated, sodium salicylate blocked COX-2 gene expression and attenuated 10,12 CLA-mediated inflammatory gene expression in epididymal WAT (Figure 5.8). However, it did not prevent 10,l2 CLA from decreasing body fat (Figure 5.3), increasing markers of browning in epididymal or inguinal WAT (Figure 5.5) or defending body temperature under cold exposure (data not shown). Collectively, these data advance the hypothesis that the induction or activation of COX-2, NF-κB or other inflammatory-related markers may be a consequence, rather than a mediator, of 10,12 CLA-activated browning.

Consistent with the above hypothesis, Ye et al. (36) recently demonstrated that browning in WAT can be activated independent of β-adrenergic signaling. For example, after 20-h exposure to 10°C, UCP1, type II iodothyronine deiodinase and PPARγ coactivator 1 mRNA levels were not completely diminished compared to controls in inguinal WAT in mice lacking β-adrenergic receptors, although their expression levels were lower than in wild type mice (36). These data provide further support for the hypothesis that WAT has the capacity to sense changes in external temperatures and respond to cold stimulus by uncoupling respiration from phosphorylation in mitochondria.

In our study, we detected significant body fat loss after 6 weeks of 10,12 CLA feeding, which was coincident with relatively lower, but not statistically significant basal body temperature (Supplemental Figure 5.1) and impaired ability to maintain body temperature when exposed to cold environment, compared to its HL control (Figure 5.4). A similar inability to regulate body temperature with 10,12 CLA supplementation during cold exposure was observed at an earlier time point (i.e., after 3 weeks of 10,12 CLA supplementation; Figure 5.4A). However, we did not measure the body fat percentage during that week. Interestingly, CLA supplementation (i.e., 1%; equal mixture of cis-9, trans-11 and 10,12 isomers, aka mixture CLA isomers) induced heat loss in C57Bl/6J mice, which coincided with significant loss of WAT mass on day 9 of treatment (37). Consistent with these data, mixed CLA isomers increased energy expenditure and tended to lower respiratory quotient in AKR/J mice after weeks 6 of treatment (38), suggesting that CLA promoted fat oxidation.

Although 10,12 CLA did not increase UCP1 mRNA or protein in BAT (data not shown), or cytochrome c oxidase in muscle (an indicator of shivering thermogenesis; data not shown) after 7 weeks of treatment, it is possible that BAT thermogenesis could be initially up-regulated, because BAT mass was decreased by 50% following 10,12 CLA treatment for 7 weeks (Figure 5.3). As BAT mass becomes compromised, WAT browning could be induced to provide heat to maintain body temperature. Thus, it is possible that BAT thermogenesis could have been initially up-regulated by 10,12 CLA, and as it became depleted, browning of WAT was up-regulated in an attempt to maintain body temperature when cold challenged. Future time course studies will need to be conducted to examine this hypothesis.

10,12 CLA-mediated loss of body fat does not appear to be mediated by adipocyte apoptosis

Another proposed mechanism by which CLA reduces body fat is via activation of apoptosis (reviewed in Ref. (4)). Supplementation of 1% mixed CLA isomers induced apoptosis in WAT in female C57BL/6J mice (39) and mixed strains of mice (37) within 1 week of treatment. Several apoptotic-related genes such as those in the TNF family, cell death factors and antiapoptotic genes were affected after 1 day of mixed CLA isomer feeding in WAT depots of male C57BL/6J mice (28). Strikingly, mice from various genetic lines responded to 1% mixed CLA isomers or 0.5% 10,12 CLA differently (37). For example, mice from low energy expenditure strains, but not from high energy expenditure lines, exhibited apoptosis in retroperitoneal WAT from either CLA treatment. Our study used a low dose of 10,12 CLA (0.1%) in male Sv129 mice for 7 weeks and did not find a consistent pattern of 10,12 CLA-mediated apoptosis in epididymal WAT depot (Supplemental Figure 5.2). We speculate that this lack of induction of apoptotic genes may be due to the higher basal metabolic rate background of Sv129 compared to the C57BI/6J strain (40).

10,12 CLA causes hepatomegaly, which is not attenuated by exercise

Steatosis has been a notorious side effect associated with consuming high doses of CLA (32,39,41). To our knowledge, there are no studies examining the ability of exercise to prevent or treat CLA-mediated steatosis. Several studies reported that exercise successfully prevented high fat-induced steatosis in rodents (28,42). For example, high fat (45% fat)-fed male C57BL/6 mice had elevated liver TG content at weeks 6 (28). Exercise (treadmill, 40 min/day, 12 m/min, 12% grade, 5 day/week) for 6

weeks prevented this high-fat diet-mediated steatosis. This same intensity and duration of exercise also have been shown to improve blood lipid profiles and enhance oxidative capacity in leg muscles of male C57BL/6 mice (29). Therefore, we adopted this exercise protocol for our study. However, exercise did not further attenuate 10,12 CLA-mediated (i) reduction of adiposity (Figure. 5.3), (ii) increase of liver weight (Figure. 5.9), or (iii) inflammatory gene expression in WAT (Figure 5.8) in Sv129 mice. Furthermore, exercise did not affect 10,12 CLA-induced browning in WAT (Figure 5.5). This could be due to different metabolic rates of these two strains or that the exercise protocol was not of sufficient intensity or duration.

Limitations

One major limitation of this study is that we did not measure energy expenditure, and thus, we do not know if 10,12 CLA decreases body fat via increased heat loss. Another limitation is that we did not measure body fat percentage and body temperature of mice exposed to 4°C during the first few weeks of the study. A third limitation is that the DEXA instrument has not been validated for mice by the manufacturer. However, we compared the actual body weights (recorded weekly) to the DEXA instrument reads (bone mass + fat + lean tissue mass). Correlation analysis was conducted by SPSS (version 20). The Person's correlation value is 0.938, and the significant Pb.001 (data not shown). Lastly, we do not know if a low dose of 10,12 CLA reduces body fat in overweight mice consuming a high-fat diet. Thus, future studies need to address these issues so as to gain further insights into the exact mechanism by which 10,12 CLA decreases body fat.

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Supplemental Table 5.1 Diet Formulations*

Ingredients (gram/kg)	LF	HF	LF+C	LF+SS	LF+SS+C
Casein	190	221	190	189	189
L-Cystine	3	3	3	3	3
Corn starch	480	218	480	478	478
Maltodextrin 10	118	115	118	118	118
Fructose	0	5	0	0	0
Dextrose, anhydrous	0	5	0	0	0
Sucrose	65	148	65	65	65
Cellulose	47	55	47	47	47
Soybean oil	24	49	23	24	23
Butter	0	31	0	0	0
Lard	19	34	19	19	19
Shortening	0	53	0	0	0
CLA, tran-10,cis-12	0	0	**1**	0	**1**
Mineral mix, S10026	9	11	9	9	9
DiCalcium phosphate	12	14	12	12	12
Calcium carbonate	5	6	5	5	5
Potassium Citrate, 1 H_2O	16	18	16	16	16
Vitamin mix, V10001	9	11	9	9	9
Choline Bitartrate	2	2	2	2	2
Sodium salicylate	0	0	0	**4**	**4**
Total	1000	1000	1000	1000	1000

*LF, low fat; HF, high fat; LF+CLA, low fat+0.1% trans-10,cis-12 conjugated linoleic acid; LF+SS, low fat+4 g/kg sodium salicylate; LF+SS+CLA, low fat+4 g/kg sodium salicylate+0.1% trans-10,cis-12 conjugated linoleic acid.

Supplemental Table 5.2 Food Intake, Body Weight, Fasting Blood Glucose, and Glucose Tolerance Test (GTT) Area Under the Curve (AUC) at the End of the Study (week 11-12)*

	LL	HH	HL		HLE		HLS	
CLA	-	-	-	+	-	+	-	+
Food Intake (g/cage)	444 ± 4.7	459 ± 18.9	457 ± 10.5	440 ± 8.1	422 ± 6.3	440 ± 9.9	440 ± 7.4	442 ± 13.3
Body weight (g)	25.7 ± 0.6^{bc}	32.7 ± 1.4^{a}	27.8 ± 0.7^{b}	25.8 ± 0.5^{bc}	25.3 ± 0.5^{bc}	24.0 ± 0.4^{c}	26.5 ± 0.6^{bc}	25.0 ± 0.9^{bc}
Fasting blood glucose (mg/dL)	60.8 ± 2.7^{c}	94.0 ± 4.4^{a}	76.5 ± 3.8^{bc}	79.3 ± 3.8^{ab}	75.9 ± 2.2^{bc}	79.1 ± 2.2^{ab}	78.7 ± 3.0^{b}	71.8 ± 4.5^{bc}
GTT AUC	17242 ± 763^{b}	25649 ± 1163^{a}	17058 ± 775^{b}	19616 ± 699^{b}	17507 ± 767^{b}	19419 ± 267^{b}	19458 ± 674^{b}	17880 ± 876^{b}

*LL, low fat diet throughout the 12 wk study; HF, high fat diet throughout the 12 wk study; HL, high fat diet for the first 5 wk and then switched to the low fat diet for the following 7 wk; HLE, high fat diet for the first 5 wk, and then switched to the low fat diet with daily exercise for the following 7 wk; HLS, high fat diet for the first 5 wk, and then switched to the low fat diet with 4 g/kg sodium salicylate for the following 7 wk; CLA, 0.1% trans-10,cis-12 conjugated linoleic aicd.

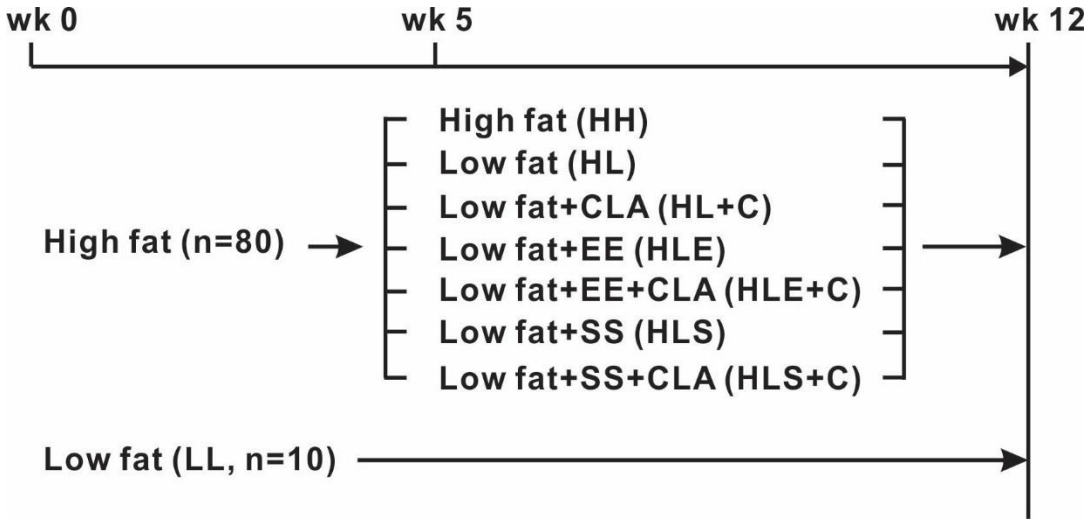


Figure 5.1 Experimental Design. Eighty male Sv129 mice were fed a high-fat diet for 5 weeks and subsequently maintained on the high-fat (HH; n=10) diet or switched to a (i) lowfat (HL) diet with (HL+CLA; n=12) or without 0.1% 10,12 CLA (n=10), (ii) low-fat diet plus exercise (HLE) with (HLE+CLA; n=12) or without 0.1% 10,12 CLA (n=12), (iii) or low-fat diet plus 4 g/kg sodium salicylate (HLS) with (HLS+CLA; n=12) or without 0.1% 10,12 CLA (n=12) for another 7 weeks. Ten mice were fed a low-fat diet throughout the study (LL).

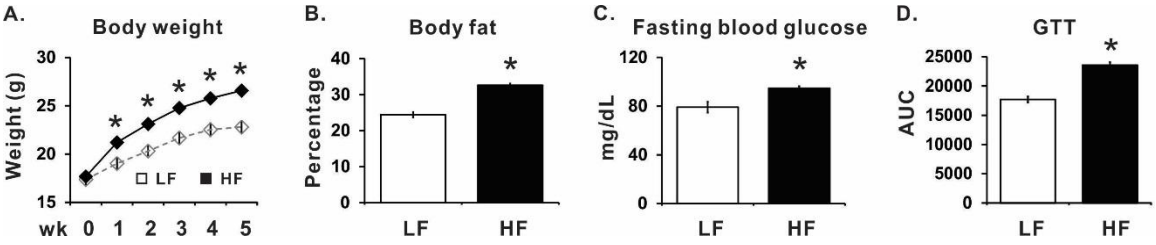


Figure 5.2 High-Fat Diet Increases Body Weight, Body Fat and Blood Glucose Level. (A) Body weights of mice fed the high-fat (HF) diet (n=80) or the low-fat (LF) diet (n=10) for 5 weeks. (B) Body fat percentages of mice that were randomly selected from HF (n=10) or the LF diet (n=10) for 5 weeks. (C) Fasting blood glucose level from mice that were randomly selected from HF diet group (n=10) or the LF diet group (n=10). (D) AUC from an intraperitoneal GTT from mice that were randomly selected from HF diet group (n=10) or the LF diet group (n=10). Mean±S.E.M. having an asterisks (*) are significantly different using the Student's t test (Pb.05).

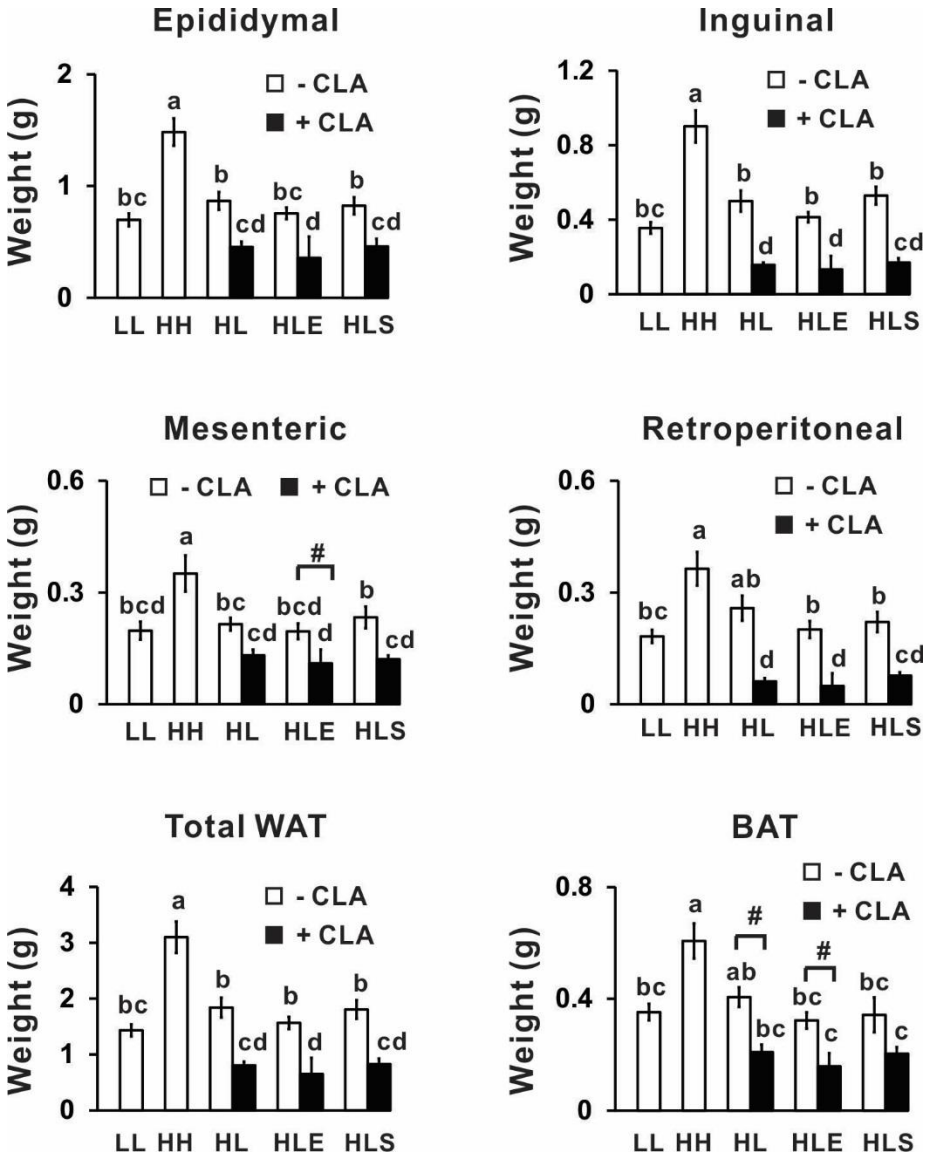


Figure 5.3 10,12 CLA Decreases Adiposity. At weeks 12, epididymal, inguinal, retroperitoneal and mesenteric WAT and BAT were excised and weighed. Means±S.E.M. (n=10–12) not sharing a common letter differ (Pb.05) by one-way ANOVA. Means±S.E.M. sharing the symbol # differ using the Bonferroni's adjustment (Pb.01). Weight of total WAT is the combined weights of the epididymal, inguinal, retroperitoneal and mesenteric depots; LL, low fat throughout the 12-week study; HH, high fat throughout the 12-week study; HL, high fat for the first 5 weeks and then switched to low fat for the following 7 weeks; HLE,

high-fat diet for the first 5 weeks and then switched to low-fat diet with daily exercise for the following 7 weeks; HLS, high-fat diet for the first 5 weeks and then switched to low-fat diet with 4 g/kg sodium salicylate for the following 7 weeks; CLA, 0.1% 10,12 CLA.

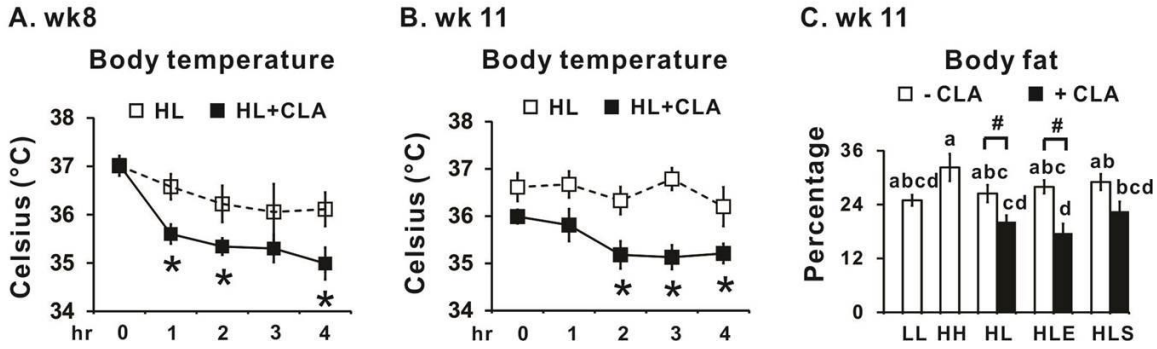


Figure 5.4 10,12 CLA Decreased Body Temperature During Cold Exposure. At weeks 8 (A) and weeks 11 (B), 10 mice from HL and HL+CLA groups were exposed to 7°C for 4 h. Body temperatures were recorded at baseline and after 1, 2, 3 and 4 h of cold exposure. (C) Body fat percentages using DEXA were measured in mice from all treatments (n=10–12 per group) at week 11. Means±S.E.M. having an asterisks (*) are significant different using the Student's t test (Pb.05). LL, low fat throughout the 12-week study; HH, high fat throughout the 12-week study; HL, high fat for the first 5 weeks and then switched to low fat for the following 7 weeks; HLE, high-fat diet for the first 5 weeks and then switched to low-fat diet with daily exercise for the following 7 weeks; HLS, high-fat diet for the first 5 weeks and then switched to low-fat diet with 4 g/kg sodium salicylate for the following 7 weeks; CLA, 0.1% 10,12 CLA.

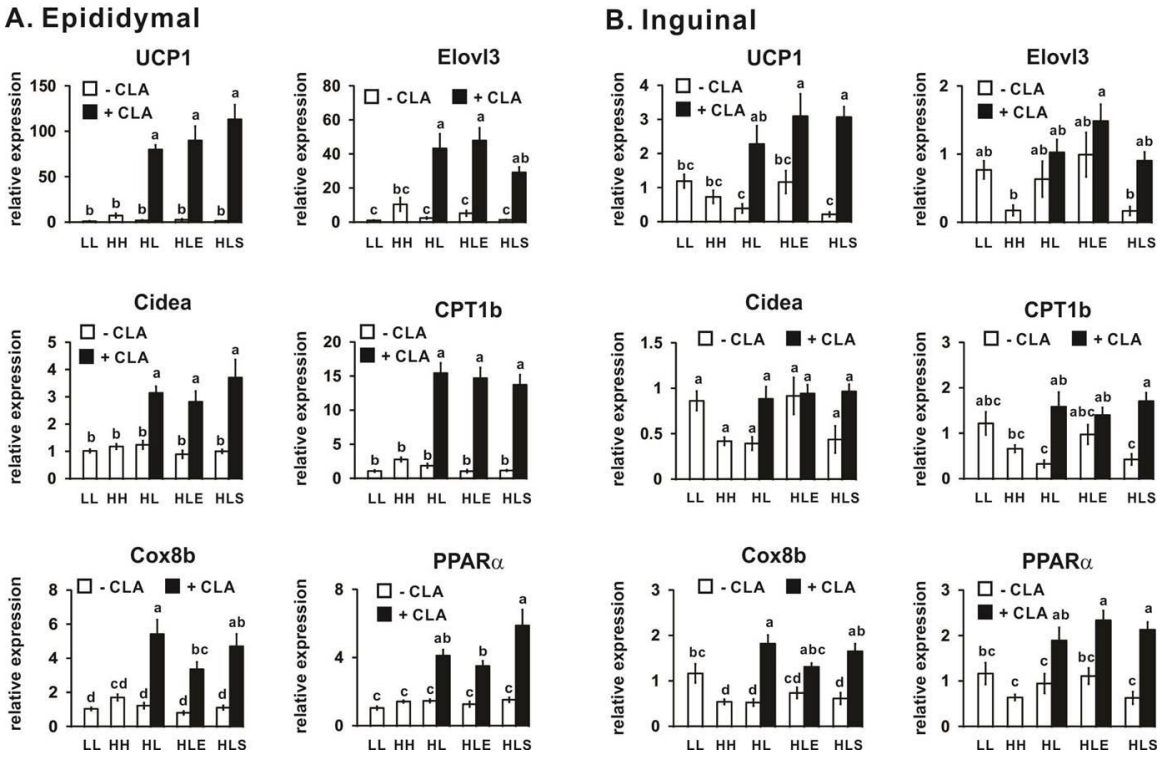


Figure 5.5 10,12 CLA Increases the mRNA Levels of Markers Associated with Browning in Epididymal (A) and Inguinal (B) WAT. mRNA levels were measured by real-time qPCR. Means±S.E.M. (n=10–12) not sharing a common letter differ (Pb.05) by one-way ANOVA. UCP1, uncoupling protein 1; Elovl3, elongation of very long chain fatty acids 3; Cidea, cell death-induced DNA fragmentation factor-a-like effector A; CPT1b, carnitine palmitoyltransferase 1b; COX8b, cytochrome c oxidase subunit VIII b; PPARα, proliferator-activated receptor α; LL, low-fat diet throughout the 12-week study; HH, high-fat diet throughout the 12-week study; HL, high-fat diet for the first 5 weeks and then switched to low-fat diet for the following 7 weeks; HLE, high-fat diet for the first 5 weeks and then switched to low-fat diet with daily exercise for the following 7 weeks; HLS, high-fat diet for the first 5 weeks and then switched to low-fat diet with 4 g/kg sodium salicylate for the following 7 weeks; CLA, 0.1% 10,12 CLA.

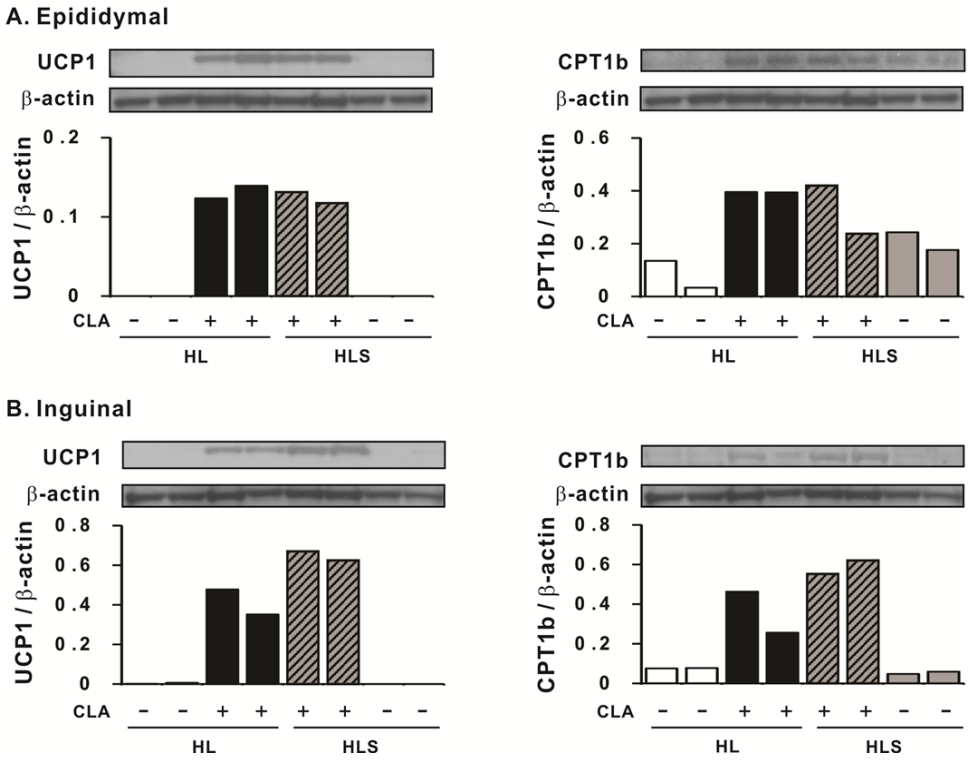


Figure 5.6 10,12 CLA Increases the Protein Levels of UCP1 and Carnitine Palmitoyltransferase 1b (CPT1b) in Epididymal (A) and Inguinal (B) WAT. Protein levels were measured by immunoblotting. β-actin was used as a loading control. Two samples from HL, HL+CLA, HLS and HLS+CLA treatments were randomly selected and ran on the same gel. We chose the most representative blots. LL, low-fat diet throughout the 12-week study; high-fat, high-fat diet throughout the 12-week study; HL, high-fat diet for the first 5 weeks and then switched to low-fat diet for the following 7 weeks; HLS, high-fat diet for the first 5 weeks and then switched to low-fat diet with 4 g/kg sodium salicylate for the following 7 weeks; CLA, 0.1% 10,12 CLA.

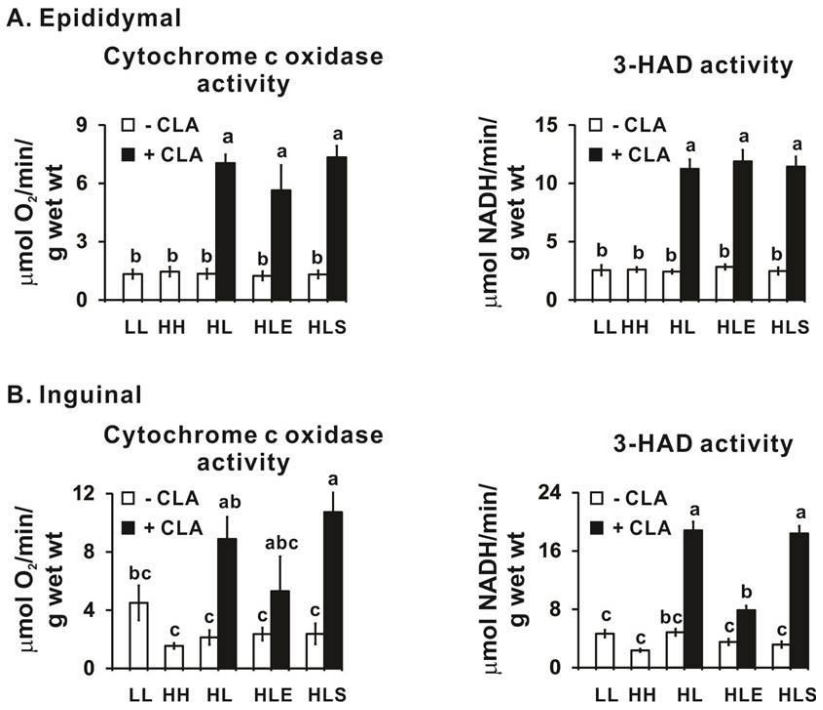


Figure 5.7 10,12 CLA Increases the Activity of Cytochrome c Oxidase and 3-HAD in Epididymal (A) and Inguinal (B) WAT. Means±S.E.M. (n=8-12) without a common letter differ ($P<0.05$) by one-way ANOVA. LL, low-fat diet throughout the 12-week study; HH, high-fat diet throughout the 12-week study; HL, high-fat diet for the first 5 weeks and then switched to low-fat diet for the following 7 weeks; HLE, high-fat diet for the first 5 weeks and then switched to low-fat diet with daily exercise for the following 7 weeks; HLS, high-fat diet for the first 5 weeks and then switched to low-fat diet with 4 g/kg sodium salicylate for the following 7 weeks; CLA, 0.1% 10,12 CLA.

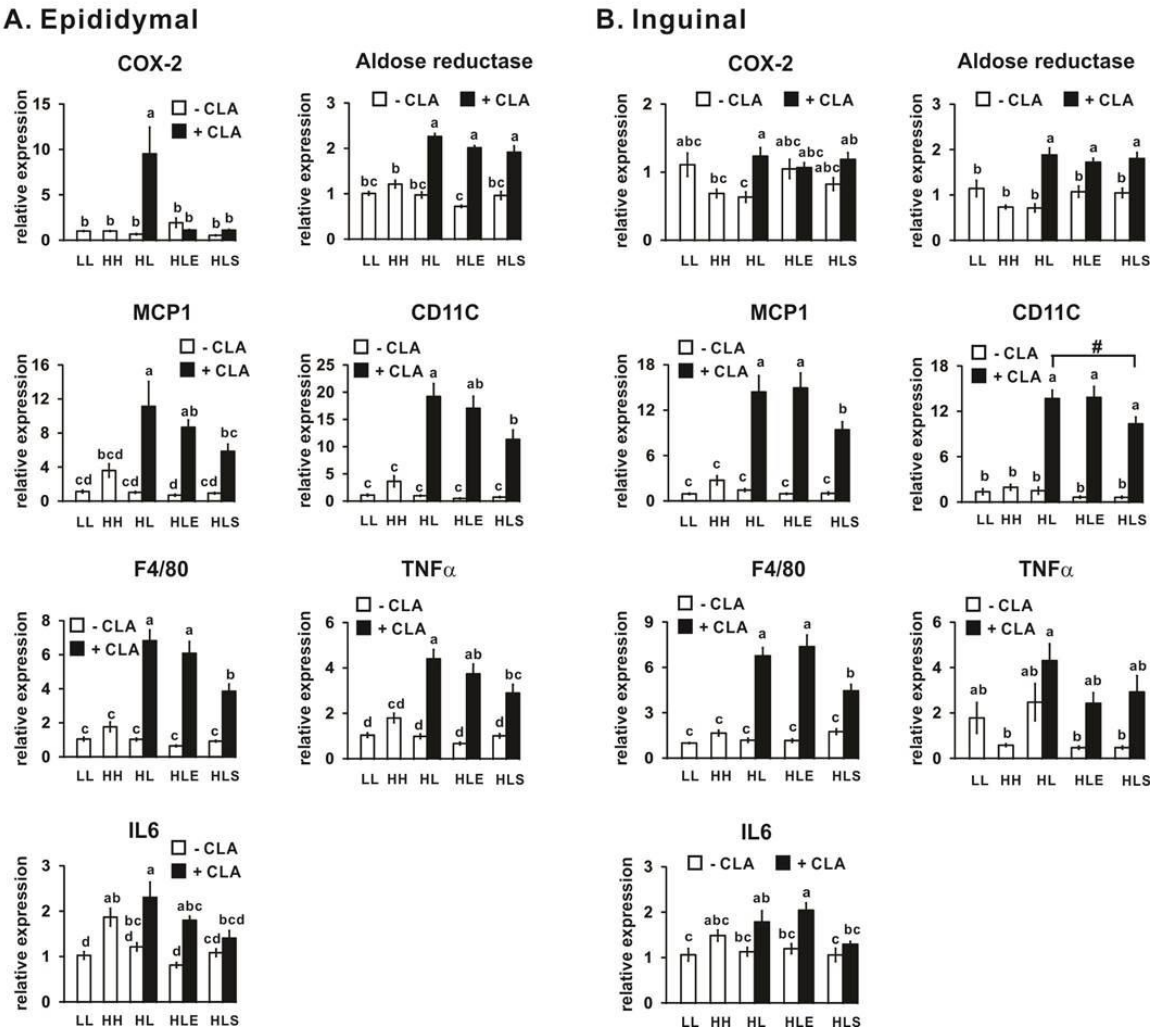


Figure 5.8 10,12 CLA Increases the mRNA Levels of Markers Associated with Inflammation in Epididymal (A) and Inguinal (B) WAT. mRNA levels were measured by real-time qPCR. Means±S.E.M. (n=10–12) without a common letter differ (Pb.05) by one-way ANOVA. Means±S.E.M. sharing the symbol # differ using the Bonferroni's adjustment (Pb.01). LL, low-fat diet throughout the 12-week study; HH, high-fat diet throughout the 12-week study; HL, high-fat diet for the first 5 weeks and then switched to low-fat diet for the following 7 weeks; HLE, high-fat diet for the first 5 weeks and then switched to low-fat diet with daily exercise for the following 7 weeks; HLS, high-fat diet for the first 5 weeks

and then switched to low-fat diet with 4 g/kg sodium salicylate for the following 7 weeks; CLA, 0.1% 10,12 CLA.

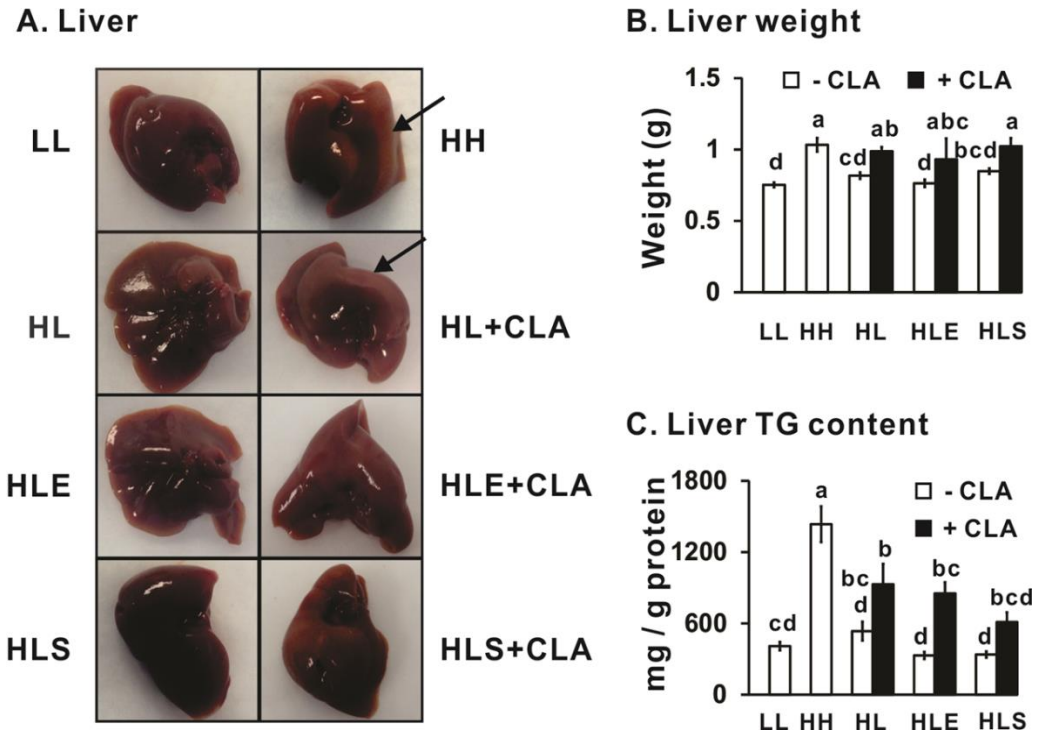
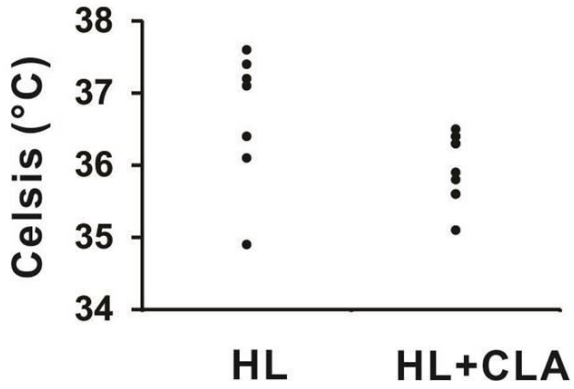
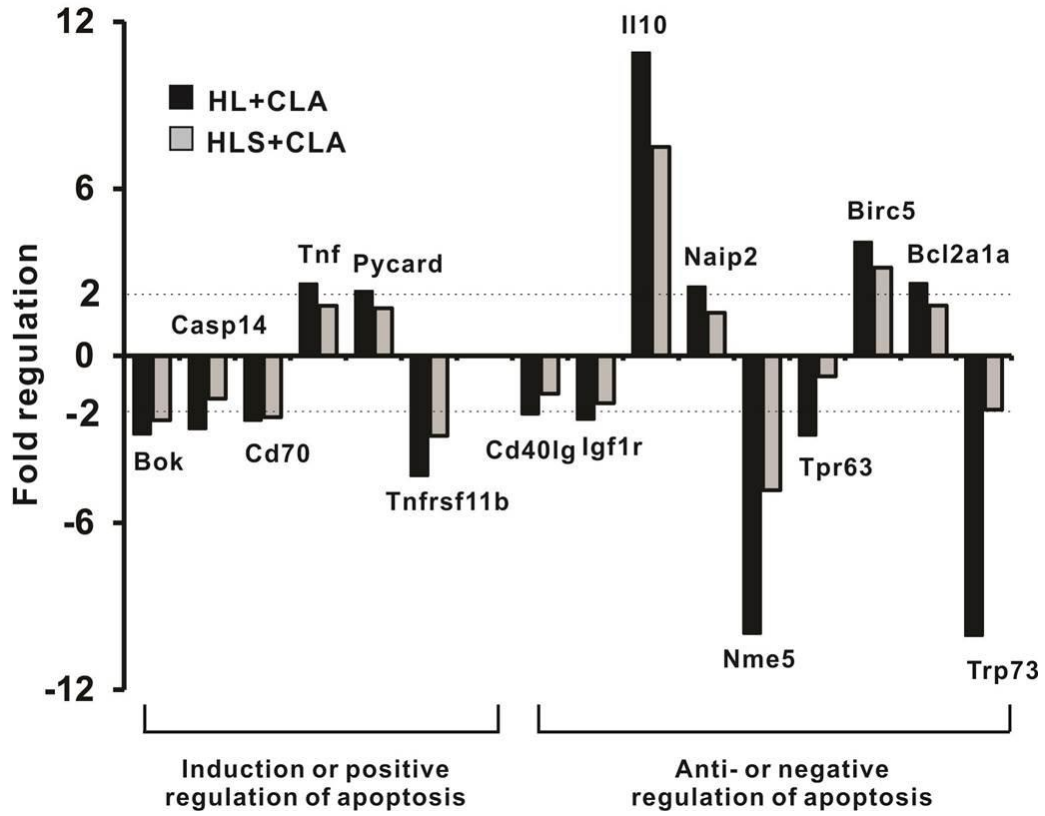


Figure 5.9 Impact of 10,12 CLA on Liver Weight and TG Content. (A) Representative pictures from each treatment group (n=6–8), with arrows pointing to areas of lipid accumulation. (B) Liver weights from each group (n=10–12). (C) Liver TG content was measured and normalized by per mg of protein (n=10–12). Mean±S.E.M. without a common letter differ (Pb.05) by one-way ANOVA. LL, low-fat diet throughout the 12-week study; HH, high-fat diet throughout the 12-week study; HL, high-fat diet for the first 5 weeks and then switched to low-fat diet for the following 7 weeks; HLE, high-fat diet for the first 5 weeks and then switched to low-fat diet with daily exercise for the following 7 weeks; HLS, high-fat diet for the first 5 weeks and then switched to low-fat diet with 4 g/kg sodium salicylate for the following 7 weeks; CLA, 0.1% 10,12 CLA.



Supplemental Figure 5.1 Body Temperature under Room Temperature at wk 11. HL, high fat for the first 5 wks and then switched to low fat for the following 7 wks; CLA, 0.1% 10,12 conjugated linoleic acid; n=10-12.



Supplemental Figure 5.2 Fold Regulation of Apoptotic Genes in Epididymal WAT. HL, high fat for the first 5 wks and then switched to low fat for the following 7 wks; HLS, high fat for the first 5 wks, and then switched to low fat with 4 g/kg sodium salicylate for the following 7 wks; CLA, 0.1% 10,12 conjugated linoleic acid. Six samples were randomly selected from each group.

CHAPTER VI

EPILOGUE

Introduction

The rising prevalence of overweight and obesity in the U.S. has burdened the country's financial budgets, medical resources, and the quality of life for overweight individuals and their families. Diminished life expectancy and obesity-related metabolic consequences including cardiovascular diseases, type 2 diabetes, respiratory complications, and non-alcoholic steatohepatitis (reviewed in 1) accompany excess body fat. Dietary supplements have become popular for weight management within the last decade. Among these supplements, conjugated linoleic acid (CLA), along with caffeine, carnitine, and green tea, are considered "fat burners", because they have been shown to increase fat metabolism and reduce body fat (reviewed in 2). However, side effects such as steatosis (3-6), insulin resistance (3-5, 7), atherogenic cholesterol profiles (reviewed in 8), and decreased leptin levels (9) have been consistently reported from supplementation of the commercially-available CLA mixture (50:50 ratio of cis-9,tran-11 (9,11) and trans-10,cis-12 (10,12) isomers) or 10,12 CLA alone in animal studies. Because of these potential side effects, the Food Standards Australia and New Zealand are considering re-evaluating the efficacy and safety of CLA consumption (reviewed in 2). Therefore, understanding the mechanisms by which 10,12 CLA decreases body fat is necessary for evaluating the safety status for commercial CLA supplements.

Our lab has previously demonstrated that 10,12 CLA increased intracellular calcium levels (10), and promoted inflammatory signals which suppressed lipid synthesis

(9, 11-14) and enhanced lipid oxidation in human primary adipocytes (10, 15). The upstream signaling cascade by which 10,12 CLA activated calcium release from the endoplasmic reticulum was my first research question (Chapter III). Concerning the CLA-mediated side effects in animals studies, I investigated the following objectives in lean or overweight male 129Sv mice: (i) the threshold dose and specific isomer that is responsible for CLA's reduction in body fat with minimum side-effects (Chapter IV); (ii) the role of inflammatory signaling in mediating CLA's anti-obesity properties (Chapter V); and (iii) the synergistic effects of CLA supplementation and exercise in reducing body fat and preventing steatosis (Chapter V).

Collectively, data from these studies indicated that: (i) 10,12 CLA activated phospholipase c, an enzyme that converted phosphatidylinositol 4,5-bisphophate to diacylglycerol and inositol 1,4,5-trisphophate, and triggered calcium release from endoplasmic reticulum and inflammatory signaling that was associated with decreased triglyceride content in human primary adipocytes; (ii) 0.1% (w/w) 10,12 CLA supplementation prevented fat accumulation without causing insulin resistance and steatosis in young 129Sv mice fed a low fat diet; (iii) 0.1% (w/w) 10,12 CLA supplementation of a low fat diet successfully reduced adiposity in overweight mice compared to mice consuming a low fat diet alone; (iv) 10,12 CLA-mediated browning and increased markers of fat oxidation in white adipose tissue (WAT) are accompanied by, and independent of, inflammatory signaling; (vi) 10,12 CLA treated mice had 50% less brown adipose tissue (BAT) than controls; and (vi) exercise did not further benefit 10,12 CLA supplemented mice from reducing body fat and other side-effects. In contrast to my hypothesis, mice fed 10,12 CLA for 5 wk had an impaired capacity to defend body temperature during cold exposure (7°C for 4 h).

However, we still do not know: (i) if 10,12 CLA supplementation will reduce body fat in overweight mice when they are continuously fed an American-type, high-fat diet; (ii) potential risks of impaired regulation of body temperature, inflammation, and steatosis due to 10,12 CLA consumption in high fat-fed mice; and (iii) potential mechanisms by which 10,12 CLA reduces body fat in high fat-fed mice. Based on these observations and remaining questions about CLA's mechanism of action, the overall objective of my future project is to determine the extent to which 10,12 CLA reduces body fat in high fat-fed mice by increasing thermogenesis in BAT and WAT, total energy expenditure, and its dependence on sympathetic activity. The central hypothesis is that 10,12 CLA quickly activates sympathetic activity, thereby increasing thermogenesis in WAT and BAT, and energy expenditure that results in impaired body temperature regulation following the loss of WAT and BAT (Figure 6.1).

Testing the hypothesis and achieving the overall objective of this project will be accomplished by focusing on the following two specific aims (Figure 6.1):

Aim 1: Determine the time-dependent extent to which 10,12 CLA reduces adiposity and increases browning of WAT and energy expenditure in overweight mice.

Aim 2: Determine the dependence of increases in WAT sympathetic activity on the anti-obesity and beiging impact of 10,12 CLA in overweight mice.

Aim 1: Determine the Time-dependent Extent to which 10,12 CLA Reduces Adiposity and Increases Beiging and Energy Expenditure in Overweight Mice

Approach

In contrast to WAT, BAT oxidizes fatty acids, mainly for thermogenesis via uncoupling phosphorylation of ATP synthesis in the mitochondria, which requires the presence of UCP-1 in the electron transport chain. The sympathetic nervous system (SNS) is the main effector to BAT thermogenesis (reviewed in 16). Stimulation by the neurotransmitter norepinephrine, β-adrenergic receptors trigger cyclic AMP release in brown adipocytes, followed by activation of protein kinase A (PKA), elevated synthesis and activity of UCP1, and lipolysis of BAT (reviewed in 16 and 17).

In response to prolonged thermogenic demands, such as a constant release of norepinephrine from sympathetic nerve fibers (reviewed in 16 and 18) or direct activation of β3-adrenoceptor on the cell membrane (19-23), mitochondria biogenesis is upregulated in subcutaneous (19-21, 23) and visceral (22) WAT. These newly “beige” cells with increased numbers and activities of mitochondria, can engage in thermogenesis by turning up fat oxidation and heat production as occurs in brown adipocytes (reviewed in 24). Another newly-discovered mechanism of WAT browning is via the recruitment of alternative macrophages including eosinophils, in local subcutaneous WAT (25-26, reviewed in 27). These circulating immune cells secrete tyrosine hydroxylase that stimulates catecholamine synthesis and encourages phenotypic changes of white adipocytes into brown adipocytes (25-26). Both mechanisms of browning have been shown to increase energy expenditure as a result (19-21, reviewed in 24, 25-26); however, the browning via the eosinophils is controversial.

I have demonstrated in the previous studies that a relative low dose (0.1%, w/w) of 10,12 CLA prevented fat accumulation in young mice fed a low fat diet for 7 wk (Chapter IV) and decreased body fat in overweight mice that were initially fed a high fat diet for 5 wk to make them fat and then switched to a low fat diet plus 10,12 CLA for 7 wk (Chapter V). This 10,12 CLA-mediated reduction of body adiposity was accompanied by an induction of mRNA levels of markers of browning (e.g., UCP1) and fatty acid oxidation (e.g., CPT1b) in the visceral (epididymal) and subcutaneous (inguinal) WAT, with a greater induction in epididymal WAT depot. I also have demonstrated that, attenuating 10,12 CLA-mediated inductions of markers of inflammation and macrophage infiltration by the chemical COX-2 inhibitor, sodium salicylate, did not prevent this 10,12 CLA's effects on browning in both types of WAT depots, even though it reduced inflammatory gene expression (Chapter VI). Notably, I observed that a reduction in body temperature by 10,12 CLA coincided with the reduction in percentage of body fat at wk 11 (i.e., after 6 wk of 10,12 CLA treatment), indicating that the loss of body fat may have impacted the mice's ability to defend body temperature during cold exposure (7°C).

Therefore, the following studies will be conducted to investigate: (i) how the amount of dietary fat would impact 10,12 CLA's effectiveness in reducing body fat in overweight mice, because the average American diet contains about 34% energy from fat with 19% of energy from saturated fats and 22% of energy from sugars (28; similar to the high fat diet we used in Chapter V), and contributes to the incidence of overweight and obesity in the U.S; (ii) how quickly 10,12 CLA induces WAT browning, and decreases the mass of BAT and WAT; and (iii) if these anti-obesity effects of 10,12 CLA are due, in part, to increased thermogenesis, lipolysis, fatty acid oxidation, and energy expenditure.

Research Design, Methods, and Analytical Procedures for Research Question 1

Study 1.1 How the dietary fat levels impact 10,12 CLA's effectiveness in reducing body fat, markers of browning and thermogenesis in overweight mice? And how quickly 10,12 CLA induces WAT browning, and decreases mass of BAT and WAT?

Design

Young, male 129Sv mice (n=120) will be fed a high fat, American type diet for 5 wk to make them overweight. Subsequently, mice will be switched to a low fat diet, or continue on this high fat diet for another 1, 3, or 7 wk without or with 10,12 CLA (0.1%, w/w) supplementation. Mice will be killed on wk 6, 8, or 12 of the study as shown in Figure 6.2.

Measurements

Body weight and food intake will be measured on a weekly basis. Basal body temperature and body temperature during cold exposure (7˚C for 4 h) from each diet group will be determined weekly by using implanted micro-transponders starting at wk 5. A dual-energy X-ray absorptiometry (DEXA) scan will be conducted on mice from each diet group at baseline (i.e., wk 5), and 2 d prior to each kill date (i.e., wk 6, wk 8, and wk 12). Intraperitoneal glucose tolerance test will be conducted at wk 5, wk 7, and wk 11. On the day of the kill, blood will be collected by cardiac puncture and used for measuring serum levels of catecholamines, free fatty acids, cytokines, and chemokines. Livers, BAT depots, and subcutaneous (inguinal) and visceral (epididymal, mesenteric, and retroperitoneal) WAT will be harvested, weighted, flash frozen in liquid nitrogen, and

stored. The entire BAT depot will be removed, separated into white and brown tissue, both types weighed, and then frozen in separate vials to distinguished mitochondrial rich, thermogenically- active BAT from stored triglyceride in BAT. The mRNA and protein levels of markers of noradrenergic activity (e.g., tyrosine hydroxylase, β3-adrenoceptor), beiging (e.g., TMEM26), browning (e.g., UCP-1), lipogenesis (e.g., PPARγ), lipolysis (e.g., HSL), and fatty acid oxidation (e.g., CPT1b), as well as several G-protein coupled receptors (e.g., GPR56 and GPRC5A were upregulated in vitro by 10,12, but not 9,11 CLA in human adipocytes; 29), in WAT and BAT will be determined by real time qPCR and immunoblotting, respectively. Another piece of WAT will be fixed for staining for mutilocular lipid droplets, UCP1, and mitochondrial abundance. Two pieces of liver will be saved for determining the triglyceride content and for histological presentation of the liver lipid content via oil red O staining.

Data analysis

Data will be analyzed using a two-way ANOVA (i.e., 4 diet types and 3 treatment durations) in a 4 X 3 factorial design by JMP 8.0 (SAS Institute Inc.) statistical software. Tukey's HSD multiple-comparison test will be used to detect significant treatment differences ($p < 0.05$). Data will be expressed as Mean ± SEM.

Study 1.2 Does 10,12 CLA increased lipid metabolism and respiration in white and brown adipocytes?

Design

Young, male 129Sv mice (n=36) will be fed a high fat, American type diet for 5 wk to make them overweight, and subsequently they will be switched to a low fat diet or continued on this high fat diet (based on the results of Study 1.1) with or without 10,12 CLA (0.1%, w/w) supplementation (Figure 6.3). The length of the study will be determined from the results of Study 1.1.

Measurements

At the end of the study, mice will be killed as described in Study 1.1. Inguinal and epididymal WAT, and BAT will be collected and pooled into threes to obtain sufficient tissue for analyses. Then, they will be digested via collagenase as described in our previous study (30). Adipocytes will be separated from stromal cells and cultured for measurements of: (i) basal and isoproterenol-stimulated lipolysis; (ii) glucose and fatty acid oxidation; (iii) markers of browning and thermogenesis; and (iv) oxygen consumption. Adenylate cyclase activity and cAMP will be measured by using bio-chemiluminescent assays from Applied Biosystems or BioTrak.

Data analysis

Data will be analyzed using a one-way ANOVA (control vs. 10,12 CLA) using JMP 8.0 (SAS Institute Inc.) statistical software. Student;s t-test will be used to detect significant ($p < 0.05$) treatment differences. Data will be expressed as Mean ± SEM.

Study 1.3 Does 10,12 CLA increase energy expenditure at basal and cold temperatures?

Design

Young, male 129Sv mice (n=20) will be fed a high fat, American type diet for 5 wk to make them overweight, and subsequently they will be switched to a low fat diet or continued on this high fat diet (based on the results of Study 1.1) with or without 10,12 CLA (0.1%, w/w) supplementation (Figure 6.4). The length of the study will be determined based on the results of Study 1.1. At the day of killing, all mice will be cold challenged at 7°C for 4 h prior to being killed to upregulate thermogenesis.

Measurements

Because we do not have the capacity at UNCG to measure energy expenditure in rodents via indirect calorimetry, this study will be conducted at the NIH-supported animal facility at the Vanderbilt University. Body temperature will be measured daily. Indirect calorimetry will be applied to assess the energy expenditure and fat oxidation under basal conditions and upon acute β-3-adrenergic stimulation (CL316,243, i.p. 0.1 mg/kg; 31) every 3 d beginning at wk 6 (i.e., the first week of 10,12 CLA treatment). Food intake and body composition will be recorded on a weekly basis. After kill, measurements will be determined based on significant results obtained from Study 1.1 and 1.2.

Data analysis

Data will be analyzed as in Study 1.2.

Expected outcomes

I expect that 10,12 CLA will increase catecholamine levels, and activate thermogenesis in BAT first. With prolonged elevation of catecholamine levels, WAT browning and thermogenesis will increase, which is associated with the inductions of markers of adrenergic activity, browning, thermogenesis, fatty acid oxidation, and lipolysis in WAT. Activation of thermogenesis will result in higher energy expenditure, and eventually decrease triglyceride storage in BAT and WAT. Once 10,12 CLA treated mice have lost a significant amount of body fat, they will have an impaired ability to maintain body temperature under basal or when cold challenged.

Potential problems and alternative strategies

I don't anticipate any problems with the proposed animal and cell culture studies, as I have experience using these models and assay protocols. In Study 1.1, I am confident that 7 wk of supplementation of 10,12 CLA will decrease body adiposity in overnight mice that are switched to a low fat diet from wk 6 (based on results presented in Chapter V). If 10,12 CLA fails to affect body composition in overweight mice that are maintain on the high fat, American type diet throughout the study, it indicates that the individuals consuming this low dose of 10,12 CLA will need to also consume a relatively low fat diet in order to lose body fat.

One limitation of Aim 1 is the measurement of SNS drive. The best indicator of SNS drive is norepinephrine turnover rate, which directly measures sympathetic drive (32-34). We chose not to include this assay in our studies because of its potential negative impact on physiological or biochemical responses, i.e., norepinephrine turnover rate assay has behavioral depressant properties.

Aim 2: Determine the Dependence of Increases in WAT Sympathetic Activity on the Anti-obesity and Beiging Impact of 10,12 CLA in Overweight Mice

Approach

Given the critical role of the SNS in regulating noradrenergic activity and body temperature, and data showing the β-3 agonists activate browning and thermogenesis in WAT and BAT (35), it is conceivable that 10,12 CLA's activation of markers of browning and thermogenesis involves, in part, the activation of the SNS. In order to investigate the dependence of SNS in 10,12 CLA mediated induction of WAT browning and decrease of body fat, I will employ chemical denervation of visceral and subcutaneous WAT and intrascapular BAT using 6-hydroxy-dopamine (6-OHDA) (36).

Another possible mechanism of WAT browning involves recruiting alternative macrophages (interleukin-4, 25) to WAT or BAT, which secretes tyrosine hydroxylase and catecholamines, at least during cold stress (25-26). However, we have demonstrated that 10,12 CLA-mediated browning of WAT is not due to macrophage infiltration, and therefore do not speculate that this is the mechanism by which 10,12 CLA activates thermogenesis (Chapter V).

Research Design, Methods, and Analytical Procedures for Research Question 2

Study 2.1 Is 10,12 CLA's decrease in body fat and beiging of WAT dependent on SNS innervation?

Design

Male 129Sv mice (n=52) will be fed a high fat diet for 5 wk as described in Study 1.1. During wk 3, all mice will be surgically exposed and injected either 6-OHDA (n=28, 10 loci with 2 μl injections of 8 mg/ml 6-OHDA with 1% ascorbic acid) or saline (n=24) in their epididymal, inguinal, and retroperitoneal WAT, and intrascapular BAT depots. After wk 5, mice will be either continued on the same high fat diet or switched to a low fat diet with or without 10,12 CLA based on the results from Study 1.1 (Figure 6.5). Although I do not have experience performing this procedure, Dr. Tim Bartness, Reagents' Professor in the Department of Biology at Georgia State University, has agreed to provide me with the appropriate training to chemically denervate WAT with 6-OHDA.

Measurements

The outcome measurements will be based on Study 1.1.

Data Analysis

Data will be analyzed using a two-way ANOVA (i.e., with or without 10,12 CLA or 6-OHDA) in a 2 X 2 factorial design by JMP 8.0 (SAS Institute Inc.) statistical software. Tukey's HSD multiple-comparison test will be used to detect significant treatment

differences ($p < 0.05$). Data will be expressed as Mean ± SEM. Considering the potential surgical complications during the study, the sample size was increased proportionally.

Expected outcomes

I expect that 6-OHDA will attenuate 10,12 CLA-mediated: (i) increase of markers of browning and thermogenesis in WAT and BAT; and (ii) reduction of adiposity.

Potential problems and alternative strategies

We will be trained by Dr. Bartness's lab group on how to treat mice with 6-OHDA. They have a wealth of experience and expertise in denervating fat depots in rodents, and therefore I do not anticipate any problems conducting this assay.

If 6-OHDA does not prevent or attenuate 10,12 CLA-mediated induction of WAT browning and reduction of adiposity, I will use β-less knockout mice obtained from Dr. Brad Lowell to examine 10,12 CLA's reduction of body fat via activation of local β-adrenergic receptors as our alternative strategy. A Material Transfer Agreement has been signed by officials at the Beth Israel Deaconess Medical Center and UNCG. This pathway does not require intact SNS for activation, thus the outcomes may differ from those examined in Study 2.1.

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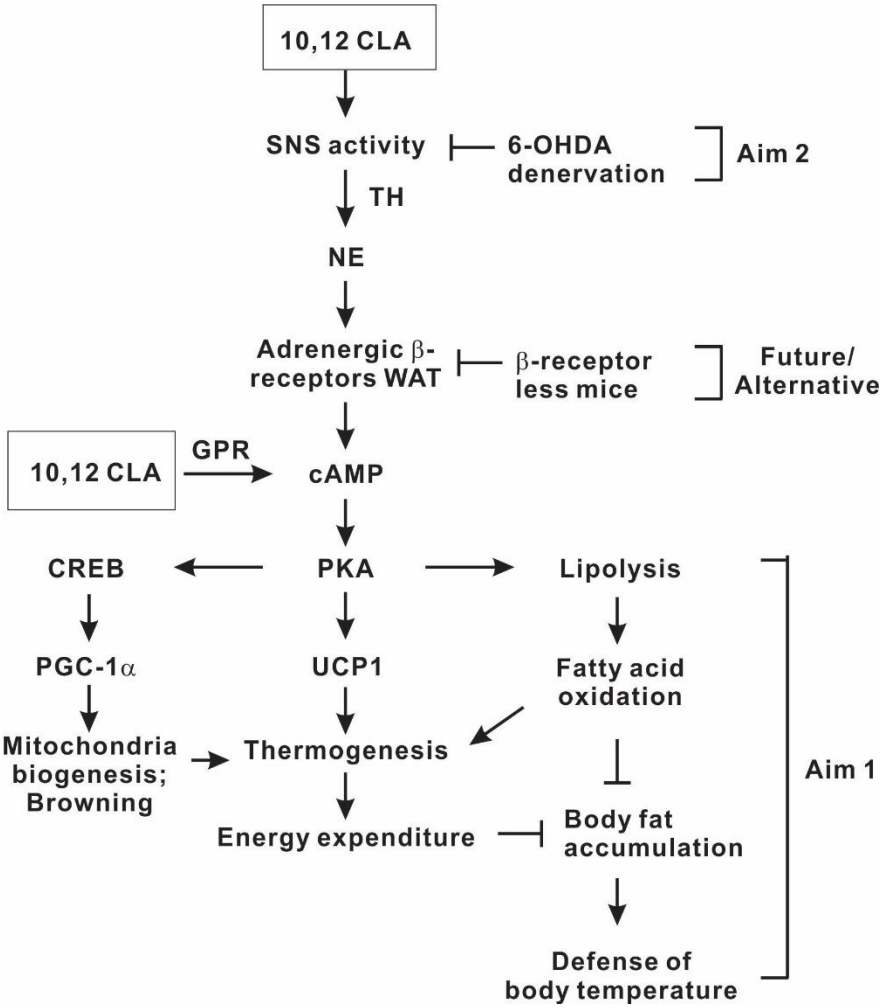

Figure 6.1 Working Model. 10,12 CLA activates sympathetic activity (Aim 2), thereby increasing lipolysis, thermogenesis, mitochondria biogenesis, and energy expenditure (Aim1), which results in decreased adiposity and impaired defense of body temperature.

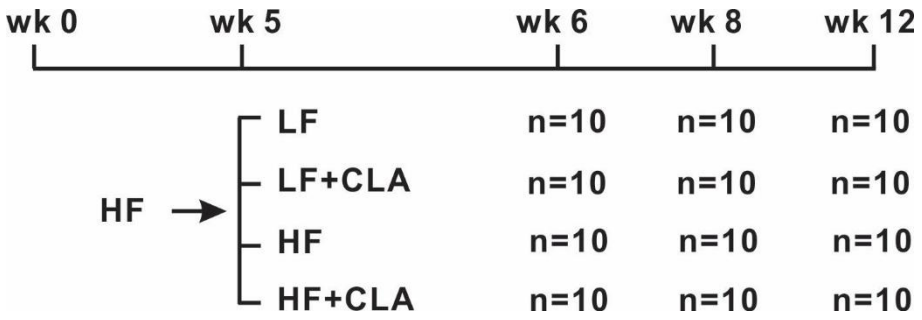

Figure 6.2 Design for Study 1.1. Young, male 129Sv mice (n=120) will be fed a high fat, American type diet for the first 5 wk. Subsequently, mice will be switched to a low fat diet, or maintained on this high fat diet, with or without supplementation of 0.1% 10,12 CLA (CLA) for another 1, 3, and 7 wk.

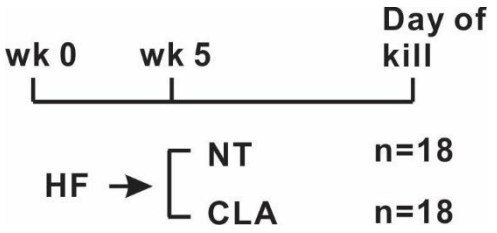

Figure 6.3 Design for Study 1.2. Young, male 129Sv mice (n=36) will be fed a high fat, American type diet for the first 5 wk. Subsequently, mice will be switched to a low fat diet, or maintained on this high fat diet based on the results of Study 1.1, with or without supplementation of 0.1% 10,12 CLA (CLA). The length of the study will be determined based on the results of Study 1.1.

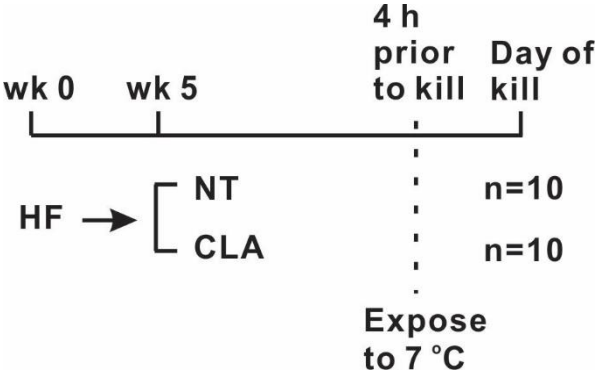

Figure 6.4 Design for Study 1.3. Young, male 129Sv mice (n=20) will be fed a high fat, American type diet for the first 5 wk. Subsequently, mice will be switched to a low fat diet, or maintained on this high fat diet based on the results of Study 1.1, with or without supplementation of 0.1% 10,12 CLA (CLA). The length of the study will be determined based on the results of Study 1.1. Mice will be challenged at 7°C for 4 h prior to kill.

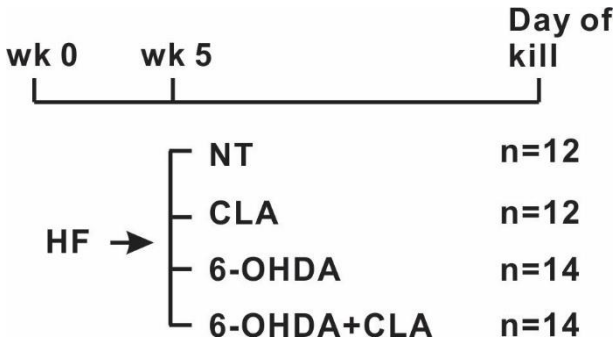

Figure 6.5 Design for Study 2.1. Young, male 129Sv mice (n=52) will be fed a high fat, American type diet for the first 5 wk. At wk 3, all mice will be injected with 6-hydroxy-dopamine (6-OHDA) or saline control. After wk 5, mice will be switched to a low fat diet, or maintained on this high fat diet based on the results of Study 1.1, with or without supplementation of 0.1% 10,12 CLA (CLA). The length of the study will be determined based on the results of Study 1.1.